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The Journal of Clinical Investigation

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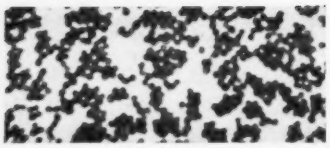

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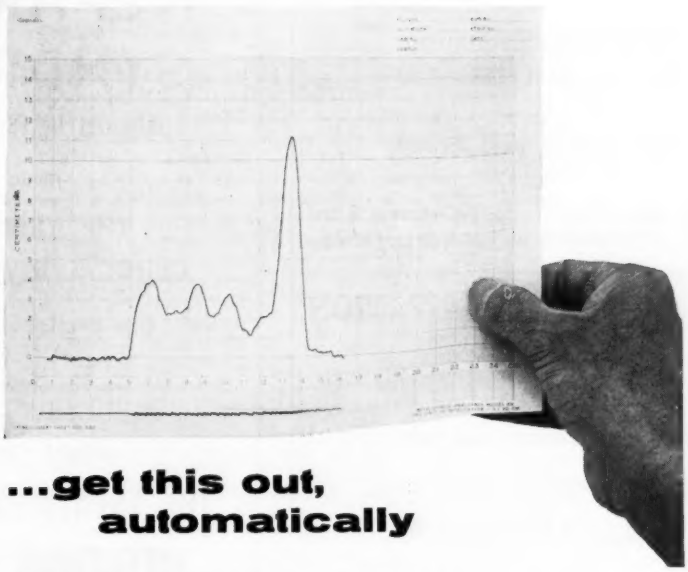

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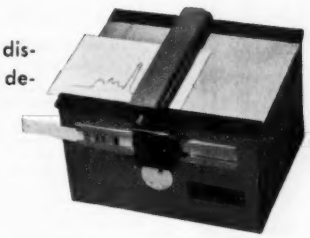

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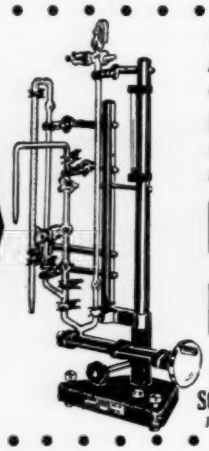

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1. J. Biol. Chem. *218*:335 (Jan.) 1956.

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2. Smith, H. W.: The Kidney, New York, Oxford University Press, 1951, p. 233.

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3. New York State J. Med. *55*:489 (Feb. 15) 1955.

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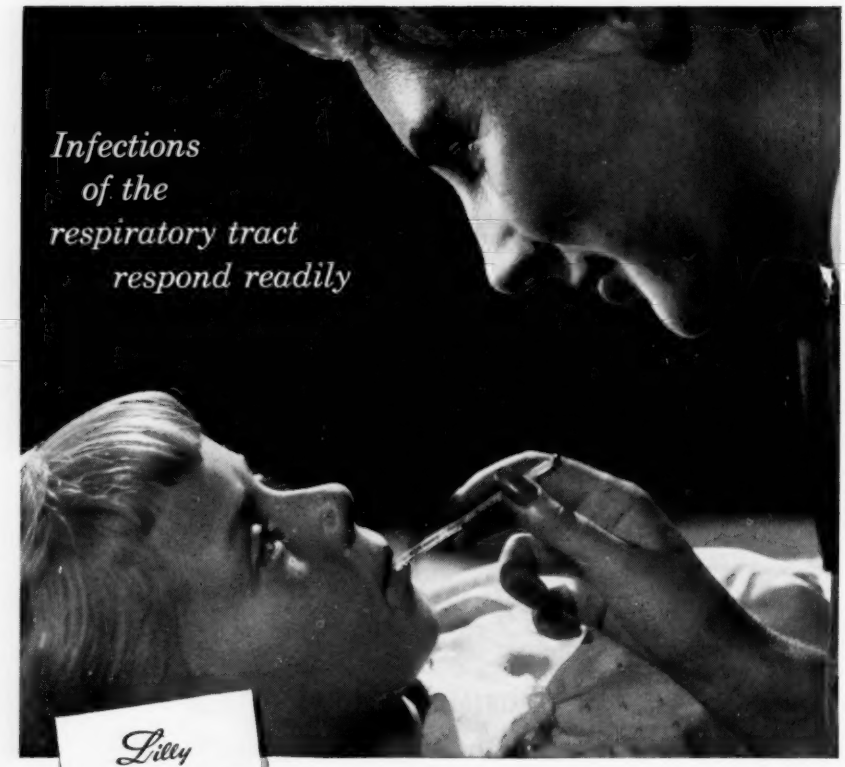

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STUDIES ON THE CLOT-PROMOTING ACTIVITY OF GLASS

By ELEAZAR SHAFRIR and ANDRE DE VRIES [1]

(From the Department of Clinical Research, Hebrew University-Hadassah Medical School, Jerusalem, Israel)

(Submitted for publication February 21, 1956; accepted March 28, 1956)

Although the clot-promoting activity of foreign surfaces has been the subject of many studies during almost a century (1) its nature is not yet understood. While glass has been thought to activate platelets (2), recent studies have mostly been concerned with the effect of glass on plasma clotting factors.

Quick maintained that contact with glass activates the precursor of prothrombin, prothrombinogen (3) as well as the precursor of plasma thromboplastin, possibly antihemophilic factor A (4). Conley and his associates (5–8) have reported that contact with glass markedly shortens the clotting time of silicone prepared "platelet free" plasma and increases its prothrombin utilization. They concluded that glass activates "plasma thromboplastin," a constituent residing in the globulin fraction. Biggs, Douglas, and Macfarlane (9) stated that the increased clotting activity developed in plasma by contact with glass is probably due to a change in Christmas Factor (PTC, antihemophilic factor B). Alexander, de Vries, and Goldstein (10) observed that contact with glass during clotting of blood favors the evolution of SPCA (convertin, Factor VII). Rapaport, Aas, and Owren (11) showed that exposure to glass markedly increases the activity of proconvertin as well as of PTC. Other communications (12, 13) attributed the clot-promoting activity of glass to removal of lipid or protein inhibitors of the first phase of coagulation, *i.e.*, the evolution of thromboplastic activity.

In the present study it is shown that a plasma clotting factor other than those mentioned above may be activated by contact with glass.

MATERIALS AND METHODS

Collection of blood. Human venous blood was collected by the two-syringe method using silicone [2] coated needles and glassware.

Native "platelet free" (PF) plasma. Blood collected into a syringe chilled by an ice jacket was centrifuged at 4° C for 10 minutes at 14,000 g in a Servall angle centrifuge. The supernatant plasma was separated and used immediately.

Oxalated "platelet free" (PF) plasma. One part of blood was mixed in a centrifuge tube with 19 parts of 0.2 M sodium oxalate. After chilling on ice for 5 minutes the blood was centrifuged at 4° C for 15 minutes at 14,000 g. The supernatant plasma was separated and used within an hour. Platelet counts performed on the undiluted supernatant by the method of Conley, Hartmann, and Morse (5) showed that the number of platelets rarely exceeded 20 per $mm.^3$ (mean ± S.D. of 40 plasmas was 14 ± 12, range 0 to 56 per $mm.^3$)

"Platelet free" (PF) $BaSO_4$-plasma (prothrombin-, proconvertin-, and PTC-free plasma). Finely powdered $BaSO_4$ (Merck) was suspended in oxalated PF plasma, 50 mgm. per ml. The suspension was kept at 20° C for 20 minutes and stirred from time to time; subsequently the $BaSO_4$ was sedimented by centrifugation for 10 minutes at 10,000 g and 4° C.

Isolation of platelets. One part of blood was mixed with 9 parts of a solution of 1 per cent EDTA [3] and 1 per cent Triton [4] in 0.7 per cent NaCl and chilled on ice. All subsequent procedures were carried out at 4° C. Platelet rich plasma was obtained by centrifugation of the blood at 500 g for 10 minutes and platelets were sedimented from this plasma by a second centrifugation for 5 minutes at 8,000 g. The sedimented platelets were rinsed and carefully resuspended in a washing fluid containing 0.05 per cent EDTA, 0.05 per cent Triton and 0.02 M sodium acetate in 0.7 per cent NaCl. The suspension was again centrifuged at 8,000 g for 5 minutes and the washing procedure repeated. The platelets were finally resuspended in 0.9 per cent NaCl and counted by the method of Rees and Ecker (14). Phase microscopic examination showed the platelets to be morphologically intact.

Activation with glass powder. Pieces of clean Pyrex glass were crushed in a mortar to fine powder. The specific surface area of the powder was 2,500 $cm.^3$ per gm. as determined with the methylene blue absorption method (15). Various plasmas were added to glass powder in a siliconed test tube as described in the results. The mixtures were kept at room temperature (20 to 22° C),

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[2] A 3 per cent solution in CCl_4 of DC 200 Silicone Fluid, Dow Corning Co., Midland, Mich., U. S. A.

[3] Ethylene Diamine Tetra-Acetic Acid disodium salt (Sequestrene), Alrose Chemical Co., Providence, N. J., U. S. A.

[4] Triton W. R. 1339, Rohm and Haas Co., Philadelphia, Pa., U. S. A.

TABLE I

The effect of contact of oxalated PF plasma with varying quantities of glass powder on the clotting time and serum prothrombin and convertin content *

Glass powder in contact with 0.4 ml. plasma (*mgm.*)	Clotting time (*min.*)		Serum prothrombin† (%)		Serum prothrombin‡ (%)		Serum convertin (%)	
	Glass removed	Glass present	Glass removed	Glass present	Glass removed	Glass present	Glass removed	Glass present
0	14		78		60		17	
2	8	7	80	70	62	40	100	123
10	8	6	75	62	64	38	120	110
50	7	4	53	42	43	30	88	57
150	6	3	45	35	40	22	63	33
300	7	2	40	20	35	16	60	10

* Four-tenths ml. oxalated PF plasma treated with glass powder was recalcified with 0.1 ml. of 0.05 M $CaCl_2$, either after removal of the glass or in its presence.
† Method of Rosenfield and Tuft (16).
‡ Method of Owren and Aas (17).

shaken gently by hand from time to time and after 10 minutes of contact centrifuged for 10 minutes at 3,000 g. In the different experiments either the supernatant glass activated plasma was transferred to another siliconed tube, or the glass was left suspended in the plasma in the original tube.

Clotting times were measured at 37° C in *siliconed* test tubes, internal diameter 11 mm.

Determination of clotting factors. Plasma and serum prothrombin were determined by the one stage procedure of Rosenfield and Tuft (16) and by the method of Owren and Aas (17). SPCA (convertin, Factor VII) in serum was assayed by the method of de Vries, Alexander, and Goldstein (18). Determination of proaccelerin (plasma Ac-globulin, labile factor) was performed by the method of Stefanini (19).

RESULTS

1. *Some factors influencing the clot-promoting activity of glass on PF plasma*

Quantity of glass; presence of glass during clotting. The effect of varying quantities of glass powder on the clotting time of oxalated PF plasma is shown in Table I. There was a marked difference in the clot-promoting effect of glass according to whether the glass was removed from the plasma before recalcification or whether the plasma was recalcified in the presence of glass. In both cases a small quantity of glass powder accelerated clotting. Larger quantities of glass powder, if present during clotting caused additional clot-acceleration but if removed before recalcification, had no such effect. In addition, with increasing amounts of glass, serum prothrombin values decreased. While with both procedures serum convertin increased markedly, additional quantities of glass powder caused a decrease in this factor. The decrease in serum prothrombin and convertin was more pronounced when glass remained during clotting.

Duration of contact with glass. In experiments in which the glass powder was removed from plasma before recalcification it was found that the clot-acceleration by a 2-minute contact was not increased by prolongation of the contact to 120 minutes.

Temperature during glass contact. Oxalated PF plasma was brought into contact with glass powder at 2°, 20°, 37° and 42° C. After 10 min-

TABLE II

Clot promoting activity induced by glass in PF plasma: Effect of decalcifying agents

Decalcifying agent	Clotting time* of DP plasma (*minutes*)	
	Without glass	Glass treated
Sodium oxalate, 0.2 M†	16	8
Sodium fluoride, 0.4 M†	18	10
Sodium citrate, 0.2 M†	16	9
EDTA, 1%‡	15	7
Amberlite IRC 50§	10	8
Dowex 50§	11	9

* The amounts of $CaCl_2$ applied were optimal for the differently decalcified PF plasmas.
† Mixed with blood in the proportion of 1 to 19. Four-tenths ml. of the derived PF plasma was recalcified with 0.1 ml. $CaCl_2$ 0.05 M.
‡ Ethylene diamine tetra-acetic acid disodium salt was added to the blood in the proportion of 1 to 9. Four-tenths ml. of the derived PF plasma was recalcified with 0.1 ml. $CaCl_2$ 0.03 M.
§ Whole blood was shaken for 10 minutes with ¼ volume of the ion exchange resin (on the sodium cycle at pH 7.7) in a siliconed Erlenmayer flask. Four-tenths ml. of the derived PF plasma was recalcified with 0.1 ml. of $CaCl_2$ 0.02 M.

TABLE III

Clotting time and residual serum prothrombin of oxalated PF plasma recalcified in the presence of glass-treated platelets*

Number of platelets per mm.³ of final clotting mixture†	Platelets							
	Whole				Frozen and thawed§			
	Without glass		Glass treated‡		Without glass		With glass	
	Clotting time (*min.*)	Serum prothrombin (%)	Clotting time (*min.*)	Serum prothrombin (%)	Clotting time (*min.*)	Serum prothrombin (%)	Clotting time (*min.*)	Serum prothrombin (%)
160,000	5	3	4.5	3	6	3	7	3
16,000	7	19	7.5	18	8	16	9	20
1,600	9	68	10	80	11	60	10	75
400	13	184	14	200	15	180	13	160
100	14	196	15	200	16	186	16	200
25	14	210	14	220	15	182	16	180
0	14	200	15	206	16	190	15	194

* Determined by the method of Rosenfield and Tuft (16). Values in excess of 100 per cent obtained with this method are discussed by de Vries, Herz, and Heiman-Hollander (20).

† One-tenth ml. of $CaCl_2$ 0.05 M added to a mixture of 0.3 ml. oxalated PF plasma and 0.1 ml. of counted platelet suspension.

‡ One ml. of suspensions containing varying number of platelets were put in siliconed tubes (internal diameter 20 mm.) containing 200 mgm. of glass beads 0.1 mm. in diameter and rotated on a disc at a slant of 45° at 20 rpm. After 15 minutes of rotation the glass beads were allowed to settle for 10 minutes and the supernatant platelet suspensions separated and used in the experiment.

§ Other platelet suspensions were frozen and thawed three times in siliconed tubes either in the presence of 200 mgm. of glass beads 0.1 mm. in diameter, or without glass beads. Supernatants were separated as above.

utes the glass was removed and the plasma recalcified at 37° C. There were no significant differences in the clotting time of plasma activated by glass at the different temperatures.

Method of decalcification of blood. Calcium was removed from blood with various decalcifying agents as indicated in Table II. In PF plasma obtained from blood treated with the calcium precipitating agents, sodium oxalate, sodium fluoride or with the chelating agents, EDTA or sodium citrate, clot-promoting activity developed on contact with glass. The decalcifying ion exchange resins Amberlite IRC 50 or Dowex 50 in contact with blood, themselves shortened the clotting time of the derived PF plasma. Contact of such plasma with glass did not result in further significant reduction of clotting time.

2. *Failure of glass to activate platelet clotting factors*

Varying quantities of platelets were treated with glass or were frozen and thawed in the presence of glass. After removal of the glass they were added to oxalated PF plasma and the recalcification time and residual serum prothrombin measured. The data in Table III show that the contact with glass did not affect the activity of platelet clotting factors.

3. *Effect of contact of native or oxalated PF $BaSO_4$- plasma with glass on clotting time, serum prothrombin and serum convertin content*

Experiments in which glass acted upon three kinds of PF plasma: native, oxalated and oxalated $BaSO_4$-treated (prothrombin-, PTC-, and proconvertin-free) are recorded in Table IV. In the case of native plasma the glass powder remained during clotting. With the oxalated plasmas, the clotting activity developed by contact with glass was tested, after removal of the glass, on oxalated PF plasma not treated with glass.

The clot-promoting activity developed by glass in PF native plasma and oxalated plasma was demonstrated by shortening of clotting time and appearance of serum convertin. On the other hand, the action of glass on the oxalated $BaSO_4$-plasma resulted in the development of clot-promoting activity with no appearance of convertin in the clotting mixture.

The serum prothrombin measured by the method of Owren and Aas (17) which gives prothrombin values independent of the variation in serum convertin content, was slightly decreased in all three kinds of glass activated plasma. Serum prothrombin values obtained by the method of Rosenfield and Tuft (16) express the combined activity of residual serum prothrombin and con-

TABLE IV

Effect of contact of native or of oxalated $BaSO_4$-treated PF plasma with glass on clotting time, serum prothrombin and convertin content

	Experiment No.*	Native PF plasma† 0.5 ml.		Oxalated PF plasma‡ 0.2 ml. + Oxalated PF plasma 0.2 ml.		Oxal. PF $BaSO_4$-plasma‡ 0.2 ml. + Oxalated PF plasma 0.2 ml.	
		Without glass	Glass treated	Without glass	Glass treated	Without glass	Glass treated
Clotting time (*min.*)	1	23	7	12	4	45	13
	2	25	4	14	7	23	10
	3	18	3	15	6	17	8
	4	19	7	17	7	19	11
	5	15	5	14	10	24	15
	6			20	8	16	10
	7			20	6	29	19
	8			14	5	30	11
Serum prothrombin§ (%)	1	120	118	82	93	20	15
	2	130	300	110	60	16	19
	3	110	160	48	20	20	17
	4	160	400	40	15	13	15
	5	90	115	100	85	52	60
	6			70	76	21	16
	7			100	150	32	22
	8			70	82	24	16
Serum prothrombin‖ (%)	1	90	60	78	36	25	14
	2	100	90	58	38	23	15
	3	100	75	44	10	24	12
	4	85	55	48	17	16	10
	5	100	100	78	40	44	40
	6			73	43	26	19
	7			100	75	36	32
	8			60	38	27	22
Serum convertin (%)	1	0	60	0	15	0	3
	2	0	80	0	25	8	8
	3	0	32	30	100	0	0
	4	0	80	25	133	0	0
	5	0	5	11	21	10	12
	6			21	54	20	10
	7			0	57	15	0
	8			27	120	30	36

* The clotting time, serum prothrombin and convertin values of the various plasmas derived from the same blood sample, are indicated by the same experiment number.

† Clotting of 0.5 ml. of native PF plasma without or with 50 mgm. of glass powder; the powder remained in contact with the plasma during clotting.

‡ Two-tenths ml. of glass activated oxalated PF- or oxalated PF $BaSO_4$-plasma from which the glass was removed, was added to 0.2 ml. of oxalated PF plasma and recalcified with 0.1 ml. of 0.05 M $CaCl_2$. For the controls, 0.2 ml. of non-activated plasma was used.

§ Method of Rosenfield and Tuft (16).

‖ Method of Owren and Aas (17).

vertin; this may explain the variable results obtained and the apparent increase in "serum prothrombin" in the experiments with native plasma.

4. *Development during contact with glass of a clot-accelerating activity in oxalated PF hemophilia A plasma rendered free of prothrombin, PTC, proconvertin, proaccelerin and fibrinogen*

The data in Table V show that glass treatment of oxalated PF hemophilia A plasma induces clot-promoting activity, which shortens the clotting time of a mixture of such plasma with oxalated PF normal plasma. For the development of the clot-promoting activity in hemophilic plasma, fibrinogen, proaccelerin, proconvertin, PTC and prothrombin are not required. This activity is not well demonstrated unless antihemophilic factor A is present in the clotting mixture. This follows from the failure of glass to shorten the clotting time significantly in most of the hemophilic PF plasma samples (native as well as oxalated) to which normal plasma had not been added.

TABLE V

Clot-accelerating activity developed by contact with glass in oxalated PF hemophilia A plasma rendered free of prothrombin, PTC, proconvertin, proaccelerin and fibrinogen

	Clotting time (*min.*)											
			Oxalated PF plasma									
	Native PF hemophilic plasma* 0.5 ml.		Hemophilic† 0.4 ml.		Hemophilic‡ 0.2 ml. + Normal 0.2 ml.		$BaSO_4$-hemophilic‡ 0.2 ml. + Normal 0.2 ml.		Stored $BaSO_4$-hemophilic§ 0.2 ml. + Normal 0.2 ml.		Heated $BaSO_4$-hemophilic‖ 0.2 ml. + Normal 0.2 ml.	
	Without glass	Glass treated	Without glass	Glass treated	Without glass	Glass treated	Without glass	Glass treated	Without glass	Glass treated	Without glass	Glass treated
Z. Z.	>180	>180	>180	>180	26	12	30	17	35	18	18	10
F. M.	>180	>180	>180	>180	50	16	84	18	65	30	28	14
N. L.			60	29	60	17	68	22	49	28		
E. S.			>180	80	20	12	45	18	18	12	34	22
Y. M.			68	30	45	19	120	64	180	72	54	32
R. F.¶												
1951	>180	>180	>180	>180	>180	>180	>180	>180	>180	>180		
1954	>180	120	>180	48	180	38	>180	88				
Y. S.¶												
1951	>180	>180	>180	>180	>180	>180	>180	>180	>180	>180	>180	>180
1954	>180	>180	>180	>180	180	24	>180	40	100	18	45	16

* Clotting of 0.5 ml. of native PF hemophilic plasma with or without 50 mgm. glass powder; the glass powder remained in the plasma during clotting.

† Clotting of 0.4 ml. of oxalated PF hemophilic plasma, recalcified with 0.1 ml. of 0.05 M $CaCl_2$. The glass powder was removed from the activated sample before recalcification.

‡ Two-tenths ml. of the various glass-activated oxalated PF hemophilic plasmas were added after removal of the glass to 0.2 ml. of oxalated PF normal plasma and the mixtures recalcified with 0.1 ml. of 0.05 M $CaCl_2$. For the controls, 0.2 ml. of non-activated samples were used.

§ Oxalated PF hemophilic plasma was stored in a siliconed tube at 4° C for 18 days. (One stage prothrombin time more than 50 seconds.) The plasma was then treated with $BaSO_4$.

‖ PF $BaSO_4$-hemophilic plasma was heated for 10 min. at 56° C. and the supernatant separated by centrifugation. The supernatant was fibrinogen-free as tested with thrombin and proaccelerin free as tested with the procedure of Stefanini (19).

¶ Both patients R. F. and Y. S. were examined twice. In 1951 both were "refractory" hemophiliacs and in 1954 both had again become responsive to normal plasma, evidenced by correction of clotting time and prothrombin consumption.

Noteworthy is the difference in the results obtained with the plasma of patients R. F. and Y. S. in 1951 and 1954. In 1951 both patients were "refractory hemophiliacs"; the prolonged clotting time and the disturbed prothrombin utilization of their blood could not be corrected by the addition of normal plasma. At that time glass treatment did not induce clot-promoting activity in their plasmas (21). In 1954 the blood of these patients had become responsive to normal plasma and paralleling this, glass treatment resulted in the appearance of the clot-promoting activity.

5. *Attempts to show adsorption of a clotting inhibitor from plasma on glass powder*

Attempts were made to elute a clotting inhibitor, possibly adsorbed on glass which had been in contact with normal or hemophilic plasma. The glass powder, after contact with a considerable amount of plasma, was extracted with small quantities of 0.9 per cent NaCl or 0.1 M $CaCl_2$ of different pH (range 4 to 12) or with ether, alcohol-ether 3 : 1, acetone or chloroform. The NaCl and $CaCl_2$ eluates were tested directly on oxalated PF normal plasma, while organic solvent extracts were evaporated and the residues resuspended in 0.9 per cent NaCl before the test. In additional experiments the eluates were incubated with diluted brain thromboplastin extract for 10 minutes at 37° C and the clotting potency of the mixture was determined by the one stage prothrombin test system. In no case, however, could a clot retarding activity be detected in these eluates.

In another experiment (Table VI) one glass powder sample was brought into contact with successive aliquots of oxalated PF normal or hemophilic plasma and the clotting time (after removal of glass) and the protein and lipid adsorbed by the glass were determined. Although the glass powder during the successive contacts with new

TABLE VI

Effect of contact of glass powder with successive aliquots of oxalated PF normal or hemophilic plasma on the clotting time and on the amount of protein and lipid adsorbed

Contacts* of glass powder with plasma aliquots	Clotting time* of plasma after contact with the glass powder sample (min.)		Protein† on the glass powder sample after contact with plasma (mgm.)		Esterified lipids‡ on the glass powder sample after contact with plasma (mgm.)	
	Normal	Hemophilic	Normal	Hemophilic	Normal	Hemophilic
No contact	15	80				
First contact	6	19	0.28	0.30	0.17	0.19
Second contact	5	16	0.30	0.35	0.16	0.17
Third contact	5	21	0.27	0.32	0.14	0.16
Fourth contact	6	24	0.32	0.30	0.15	0.18
Fifth contact	5	25	0.33	0.37	0.16	0.16

* Two-ml. aliquots of oxalated PF plasma in 10 test tubes were activated each with 500 mgm. of glass powder. After centrifugation the supernatants were separated, pooled and a 0.4-ml. sample recalcified with 0.1 ml. of 0.05 M $CaCl_2$. The glass powder from two tubes was processed as described below. To the glass powder in each of the remaining eight tubes a new 2-ml. aliquot of the original non-activated plasma was added and the above procedure was repeated each time with two tubes less.

† After each contact experiment the glass powder from one tube was washed three times with 5 ml. of 0.9 per cent NaCl following which 1 ml. of 2.5 N NaOH was added and the mixture heated on a boiling water bath for 20 minutes. The tyrosine-like activity of the supernatant was determined by the method of Herriott (22). As determined with this method the initial protein content of the normal plasma was 7.4 gm. per 100 ml., of the hemophilic plasma 7.9 gm. per 100 ml.

‡ After each contact-experiment the glass powder from the second tube was washed as above and extracted with 3 ml. of alcohol-ether mixture 3:1. Esterified lipids were determined in the extract by the method of Stern and Shapiro (23). The plasma values obtained with this method were: normal 340 mgm. per 100 ml., hemophilic 370 mgm. per 100 ml.

plasma aliquots did not lose its ability to induce clot-promoting activity, the amount of protein and lipid adsorbed by the glass did not increase above that adsorbed during the first contact.

In the experiment recorded in Table VII, one sample of oxalated PF normal or hemophilic plasma was brought into contact with successive aliquots of glass powder. The shortening of the plasma clotting time after the first contact did not become more pronounced on repeated contacts with new glass; on the contrary, the clotting time again increased. The observed decrease in plasma prothrombin accompanying successive contacts of the plasma with glass, does not seem sufficient to explain the prolongation of the clotting time. However, evidence has also been obtained that the application of excessive amounts of glass to oxalated PF plasma results in diminution of plasma proconvertin activity (24). Whether the concentration of other clotting factors such as PTC, Factor X (25), etc. is also decreased by the successive contacts with glass, is not known.

These results indicate that the development of clot-promoting activity in plasma in contact with glass is not due to removal of a clotting inhibitor by adsorption onto the glass surface.

TABLE VII

Effect of contact of a PF normal or hemophilic plasma sample with successive aliquots of glass powder on clotting time and plasma prothrombin content

Contacts* of plasma with successive aliquots of glass powder	Clotting time† after contact with glass powder (min.)		Plasma prothrombin‡ after contact with glass powder (%)	
	Normal	Hemophilic	Normal	Hemophilic
No contact	18	80	100	100
First contact	7	23	96	94
Second contact	7	19	94	94
Third contact	9	26	85	90
Fourth contact	11	35	75	82
Fifth contact	15	70	65	80

* One plasma sample was activated five times with fresh aliquots of glass powder, 250 mgm. per ml. The initial amount of plasma was 10 ml. After each contact with glass 2 ml. were removed for clotting and prothrombin tests.

† Four-tenths ml. of plasma were recalcified (after removal of glass) with 0.1 ml. of 0.05 M $CaCl_2$.

‡ Determined by the method of Owren and Aas (17).

DISCUSSION

In the experiments described above it was observed that the clotting activity of isolated platelets, or of platelet clotting factors, liberated by freezing and thawing of isolated platelets, was not enhanced by contact with glass. On the other

hand contact of "platelet free" (PF) plasma with glass induced a clot-promoting activity.

In the present study, the clot-accelerating activity induced by transient contact with glass in variously treated oxalated PF normal and hemophilic A plasmas was estimated. This was measured by the ability of such plasmas to shorten the recalcification time of mixtures of these plasmas with oxalated PF normal plasma. With this method it was possible to show that clot-promoting activity is developed by glass in PF hemophilia A plasma, rendered free of prothrombin, PTC, proconvertin, proaccelerin and fibrinogen. The clotting factor activated by glass in such plasma has not been identified. It is unlikely that the factor concerned is the "fourth thromboplastic factor" of Spaet, Aggeler, and Kinsell (26) or the Clotting Factor X (25), as these factors would have been destroyed by the heating to 56° C employed to remove fibrinogen from the test plasma. However, among other factors recently postulated to contribute to the evolution of blood thromboplastic activity, PTA (plasma thromboplastin antecedent, 27) and the "Hageman Factor" (28) might be present during activation by glass of the plasma system used here.

These observations do not disagree with the reported action of glass on proconvertin and PTC (9–11). Indeed some of them are compatible (Table IV) with the conclusions of Rapaport, Aas, and Owren (11) as to proconvertin activation by glass. Considerable serum convertin activity developed in native or oxalated PF normal plasma, on contact with glass. However when glass was applied to oxalated $BaSO_4$-treated PF normal plasma (proconvertin free), no serum convertin developed when such plasma was added after removal of glass, to oxalated PF plasma (proconvertin containing), and the mixture recalcified. In this experiment, the action of glass resulted in shortening of clotting time of the mixture without development of detectable serum convertin. Glass thus seems to act on proconvertin as well as on other, yet unidentified factor(s).

Whether the greater clot-accelerating effect of glass *present during* clotting of oxalated PF plasma, as compared with the effect of glass when *removed before* recalcification (Table I), is due to an action on proconvertin or on other plasma factors, has not been elucidated. It is pertinent that the clotting of purified fibrinogen-thrombin mixture is accelerated in the presence of suspended glass powder, while removal of the glass powder after contact with the fibrinogen before the addition of thrombin does not result in shortened clotting time (24). It is possible that the finely divided glass particles present during clotting may promote the formation of primary fibrin strands.

The observations that the residual high level of serum prothrombin remaining after clotting of PF plasma was decreased when the plasma was treated with glass before recalcification are in agreement with those of Dick, Jackson, and Conley (8). However, as in the present experiments (Table VII) it was shown that after contact with a large area of glass prothrombin may be partly removed from plasma, we hesitate to attribute the decrease in serum prothrombin after contact with glass, to enhanced prothrombin utilization.

SUMMARY

1. The effect of glass on the evolution of clot-promoting activity in "platelet free" plasma was examined. Data are presented on the influence of the amount of glass, of the duration of contact, of the temperature and of the method of decalcification of the plasma on this process.

2. Contact of glass with isolated platelets or with platelet factors liberated by freezing and thawing, did not result in increase of their clotting activity.

3. Contact with glass induced a clot-promoting activity in oxalated hemophilia A plasma, free of platelets, prothrombin, PTC, proconvertin, proaccelerin and fibrinogen.

4. Attempts to demonstrate the adsorption of a plasma clotting inhibitor onto glass were unsuccessful. Contact with large area of glass was shown to remove some prothrombin from oxalated plasma.

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STUDIES ON THE CIRCULATORY CHANGES IN THE DOG PRODUCED BY ENDOTOXIN FROM GRAM-NEGATIVE MICROORGANISMS

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A clinical problem of increasing magnitude is the occurrence of hypotension in patients with bacteremia associated with the liberation of endotoxin from gram-negative microorganisms (1–3). Although the local vascular effects of endotoxin have been extensively studied in animals, particularly with reference to necrotizing effects on tumors (4, 5) and the Schwartzman reaction (6, 7), the hemodynamic alterations produced by these bacterial products remain unexplained. Endotoxins constitute a group of similar chemical substances identified as protein-polysaccharide complexes (8). In the course of a series of investigations reported elsewhere (9, 10), it was found that the pattern of the shock and the pathological changes in the gastrointestinal tract produced by the intravenous injection of endotoxins derived from *Brucella melitensis, Escherichia coli, Serratia marcescens* [1] and *Salmonella typhosa* [1] were indistinguishable. The uniform pathophysiological changes permit the tentative assumption that various endotoxins are closely related with respect to their pharmacological activity (11).

Intravenous administration of endotoxin in suitable doses to anesthetized adult dogs regularly produces a precipitous fall in arterial blood pressure and a simultaneous rise in portal vein pressure. Very similar changes, except for quantitative differences, have been observed in the cat, the rabbit, and the rat. These changes have been traced to pooling of blood in the portal system and particularly in the liver (12). In the dog, the portal vein pressure returns to baseline levels and partial recovery of the arterial pressure occurs during the first 15 minutes after injection. Over the following 1 to 5 hours the arterial pressure again falls to lower values from which it does not ordinarily recover (10).

Since the sequence of hemodynamic events following injection of endotoxin to adult dogs was constant and reproducible, this method seemed well suited for further inquiry into the mechanism of the type of shock that is produced by these bacterial products. The present studies represent an attempt to define the initial cardiovascular alterations which are responsible for the profound blood pressure changes previously observed.

MATERIALS AND METHODS

Endotoxin. Purified endotoxin of the Boivin type derived from *E. coli* or *Br. melitensis* was used for most of the experiments. The method of preparing this type of endotoxin has been previously described (13). Washed, heat-killed *E. coli* organisms grown on "Albimi" Brucella agar medium and diluted in an equal volume of sterile saline were employed in many experiments. Earlier observations had established that this "crude" endotoxin and the highly purified endotoxins produce identical hemodynamic changes (10). In several later experiments "crude" endotoxin containing 50 mg. of dried bacterial bodies per cu. ml. was prepared from cells grown in synthetic (non-protein containing) media according to a technique described by Braude, Carey, Sutherland, and Zalesky (14). Purified *Br. melitensis* and *E. coli* endotoxin were used in a dosage of 10 and 5 mg. per kg. respectively, and "crude" endotoxins were used in a dosage of 0.3 ml. per kg. Shock was produced by a single injection given intravenously.

Procedures. Adult mongrel dogs were used and all experiments were conducted under pentobarbital sodium anesthesia except for studies of cardiac output which were done under thiopental sodium. In each case, the anesthetic was given intravenously in a dosage of 30 mg. per kg. with supplementary doses as needed. Arterial pressures were measured in the femoral or carotid artery. Portal vein pressure was measured by a catheter advanced into the vessel from a splenic branch vein. Pressures were recorded by means of Statham strain

[1] Endotoxin from *Serratia marcescens* was kindly supplied by Dr. M. J. Shear, National Cancer Institute, and endotoxin from *Salmonella typhosa* by Dr. Dennis Watson of the University of Minnesota.

gauge manometers with optimal damping and a Sanborn Polyviso recorder. Calibrations were made with a mercury manometer. Endotoxin was injected into the femoral vein or into venous tubing leading to the right atrium.

Inferior vena cava pressures either above or below the diaphragm were measured in six animals through catheters advanced from the femoral vein. Subcutaneous small vein pressure was measured in a dorsal foot vein in three dogs according to a technique described by Haddy, Richards, Alden, and Visscher (15).

Cardiac output was determined in four dogs before and at specified intervals after the injection of endotoxin using the dye-dilution technique of Nicholson and Wood (16). Evans blue dye (T-1824) was introduced into the right atrium through a catheter advanced from the jugular vein. Continuous photokymographic recordings of dye concentration in blood from the carotid artery were made using the cuvette oximeter.

Total body perfusion was performed with an oxygen-dispersion type of pump-oxygenator as described by Clark, Gollan, and Gupta (17). The system was primed with an excess of blood obtained by exsanguination of two large donor dogs. Oxygenated blood was introduced into the left subclavian artery and venous blood was picked up from the right auricle. The heart was entirely excluded from the circulation by the ligation of the pulmonary artery and the aortic root. Constant pressure perfusion was maintained in one experiment by use of a Sigmamotor pump and a Starling resistor as a shunt. The pump, maintained at constant output, was used for total perfusion experiments in two dogs.

Vascular pooling was measured in six dogs using a reservoir system which was interposed between the central ends of the great veins and the heart (Figure 1). The reservoir consisted of a glass cylinder maintained in a 37° C water bath which was placed 30 cm. below the heart level of the experimental animal. Unoxygenated blood was pumped from the base of the cylinder to the right atrium employing a Sigmamotor pump. The volume of blood in the cylinder was measured by the hydrostatic pressure of the fluid column acting on a strain gauge manometer. The atrial infusion pressure was used as a monitor. Since this pressure was created by flow through a tube to the right atrium, the pressure varied as

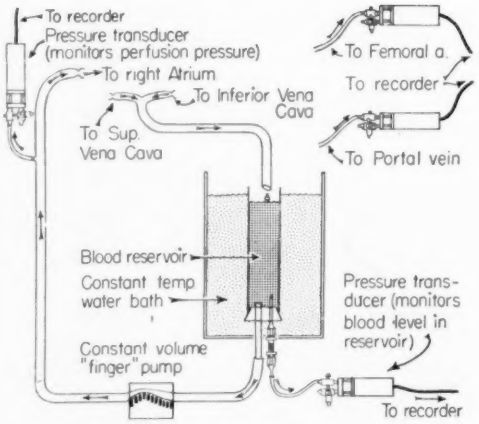

FIG. 1. ARRANGEMENT OF THE RESERVOIR SYSTEM AND THE RECORDING INSTRUMENTS FOR THE QUANTITATION OF VENOUS RETURN

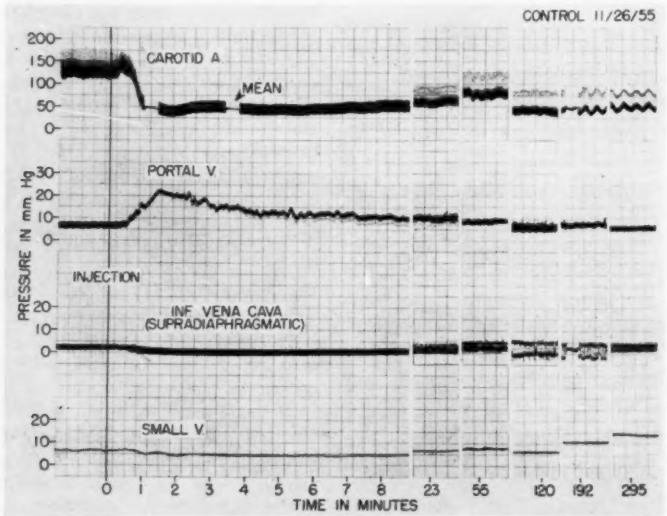

FIG. 2. ARTERIAL AND VENOUS PRESSURES FOLLOWING THE INJECTION OF ENDOTOXIN

a function of flow and resistance to filling in the right atrium. Constancy of this pressure indicated constant inflow and the absence of heart failure. The system was primed with 400 ml. of heparinized blood from a donor dog. The experimental animal was respired with air. Thoracotomy was performed by splitting the sternum. The azygos vein was ligated and 1 mg. per kg. of heparin was injected intravenously. The atrial catheter was inserted through the auricular appendage and the superior vena cava was cannulated. Infusion at a rate equal to the superior vena cava flow was begun. A catheter was subsequently introduced into the inferior vena cava so that the entire venous return passed through the reservoir. The infusion pump was gradually speeded up until an average flow of approximately 85 ml. per kg. was attained. This flow was maintained constant for the remainder of the experiment. In most instances between 100 and 300 ml. of blood were taken up from the reservoir before input and return were equal.

Serial electrocardiographic records of nine dogs were studied before and after initiation of hypotension with endotoxin. Limb lead tracings were obtained by reversing the conventional leg leads and positioning the right arm lead in the posterior neck.

RESULTS

Venous pressures

During the period when both the systemic blood pressure fell rapidly and the portal vein pressure rose, following the injection of endotoxin, the inferior vena cava pressures remained essentially unchanged (4 dogs), or decreased as much as 2 mm. Hg (2 dogs). Subcutaneous small vein pressure fell 1.5 to 7 mm. (3 dogs) during the same period. With partial recovery of the blood pressure and fall in portal vein pressure, the pressures of both the inferior vena cava and small veins returned to their previous levels (Figure 2). A small but progressive rise of small vein pressure occurred in two of the three animals after the second hour.

Cardiac output and peripheral resistance.[2]

After injection of the endotoxin there was a sharp decrease in cardiac output. With partial recovery of the arterial pressure, cardiac output was correspondingly increased (Table I). It was of considerable interest that changes in arterial

[2] The kind cooperation of Dr. E. B. Brown and the technical assistance of Mrs. Lois Trank and Mr. Richard Clancy with this group of experiments are gratefully acknowledged.

TABLE I

Serial determinations of the cardiac output and peripheral resistance in dogs after the administration of endotoxin *

Dog No.	Endotoxin	Wt. Kg.	Sex	Mean arterial blood pressure *mm. Hg*						Cardiac output *L./Kg./min.*						Peripheral resistance *dynes seconds cm.*$^{-5}$					
				Control	Minutes					Control	Minutes					Control	Minutes				
					2	10	32	53	100 173		2	10	32	53	100 173		2	10	32	53	100 173
1	*E. coli* (purified)	12.0	M	122	82	47		72	67	.113	.043	.041		.060	.057	7,200	6,980	7,570		8,000	8,000
2	*E. coli* ("crude")	14.5	M	150	47	50		52	55 62	.240	.050	.054		.038	.037 .051	3,980	5,220	5,130		8,080	7,700 6,700
3	*E. coli* ("crude")	18.6	M	175	115	88	115	55	75	.180	.097	.044	.074	.058	.050	4,170	4,440	8,530	5,840	4,075	6,450
4	*Brucella* (purified)	14.0	M	90	38	47	37	50		.156	.051	.060	.067	.066		3,300	4,220	4,475	3,150	4,350	

* The time in minutes indicates the approximate interval between injection of toxin and the measurement of cardiac output.

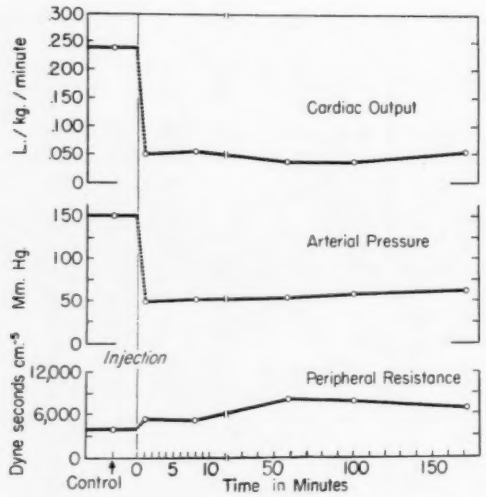

FIG. 3. RELATIONSHIP OF THE CARDIAC OUTPUT AND THE CALCULATED PERIPHERAL RESISTANCE TO ARTERIAL PRESSURE FOLLOWING INJECTION OF ENDOTOXIN

pressure during the two hours following injection were closely correlated with changes of cardiac output (Figure 3). The calculated peripheral resistance remained essentially constant or rose. In no instance was it possible to attribute the arterial hypotension to a fall in calculated peripheral resistance.

Total body perfusion

When endotoxin was injected into the animals maintained by a mechanical "heart-lung" system, no fall in arterial blood pressure was observed. On the other hand, a rise of portal vein pressure was not prevented. During constant pressure perfusion (Figure 4), the accentuated rise in portal vein pressure proved that elevation of this pressure is a basic response to endotoxin which may occur without fall in arterial pressure.

Venous return

Injection of endotoxin into dogs maintained with constant cardiac filling was shortly followed by an exaggerated and prolonged elevation in portal vein pressure. Coincident with a rise in portal vein pressure there was a rapid decline in the level of blood contained in the reservoir due to a deficient venous return. Infusion pressure and arterial pressure remained constant. Large quantities of donor blood were added to the reservoir at short intervals to compensate for the inadequate venous return. Maximum pooling occurred in the first ten minutes and corresponded to the period of exaggerated portal pressure rise (Figure 5 and Table II). Continued slower loss occurred until termination of the experiment. Although some experiments were continued for periods up to three hours, data are reported only for the first half hour. The poor condition of the preparation, in part due to the loss of blood from rupture of distended blood vessels in the viscera, made observations in the later periods of the experiment of questionable significance.

Electrocardiographic studies

The heart rate was reduced on the average from 186 to 151 beats per minute after the initiation of hypotension with endotoxin. Over the next thirty minutes the heart rate gradually returned to its former level. No cardiac arrhythmia or conduc-

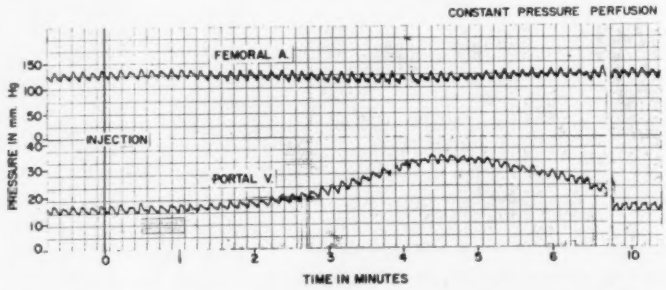

FIG. 4. PORTAL VEIN PRESSURE RESPONSE TO THE INJECTION OF ENDOTOXIN DURING TOTAL BODY PERFUSION AT CONSTANT PRESSURE

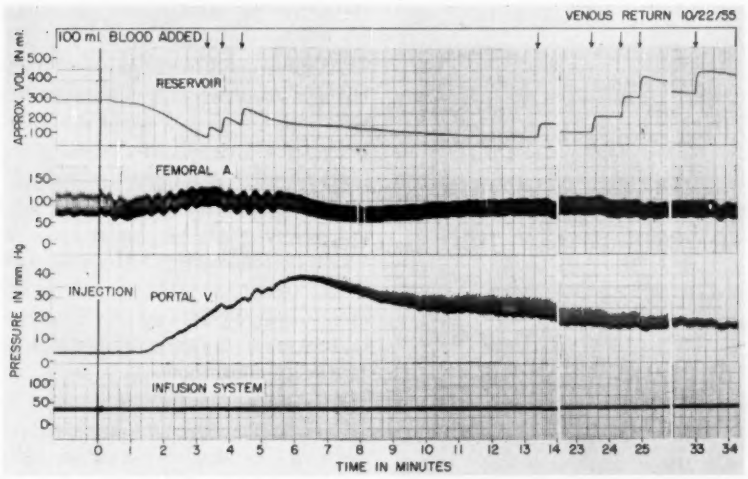

FIG. 5. RECORD OBTAINED IN A REPRESENTATIVE EXPERIMENT DURING QUANTITATION OF POOLING FOLLOWING THE INJECTION OF ENDOTOXIN

tion defect was observed in any of nine animals studied. Segments of a typical record are shown in Figure 6.

DISCUSSION

Arterial hypotension occurring in the course of an overwhelming infection or initiated by the injection of bacterial products has variously been attributed to cardiac injury (18, 19), diffuse injury to capillaries and blood vessel walls resulting in loss of vasomotor tonus or leakage (20–23), to an insult of the central nervous system (24, 25) or to a combination of two or more of such effects

TABLE II

Volume of blood pooled after injection of endotoxin

No.	Wt.	Sex	Endotoxin	Arterial blood pressure mm. Hg 0″	5″	10″	30″	Portal vein pressure mm. Hg 0″	5″	10″	30″	Volume of blood pooled ml./kg. 5″	10″	30″
1	19.0	F	*E. coli* (crude)	112	108	110	130	7	35	17	8	36	46	68
				60	78	58	58							
2	9.0	F	*E. coli* (crude)	165	195	125	135	6	50	48	13	51	82	103
				95	85	70	70							
3	9.5	F	*E. coli* (purified)	142	137	135	142	9	31	28	18	22	28	43
				102	82	85	75							
4	12.2	F	*E. coli* (purified)	120	115	97	110	4	31	24	15	30	41	51
				75	80	60	68							
5	8.0	M	(crude, synthetic)	100	100	115	130	9	43	22	7	44	66	100
				40	40	45	30							
6	7.0	F	*E. coli* (crude)	165	170	190	85	5	38	15	9	61	61	114
				100	90	100	57							
		Mean		134	138	129	122	7	38	26	12	40.7	54	79.8
				79	76	70	60							

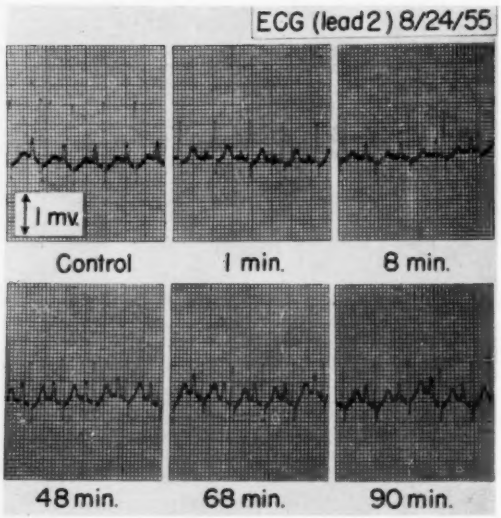

FIG. 6. SEGMENTS OF A TYPICAL ELECTROCARDIOGRAPHIC RECORD OBTAINED AT INTERVALS FOLLOWING INJECTION OF ENDOTOXIN AT 0 MINUTES

(26–28). In an earlier study we found that shock caused by endotoxin is not accounted for by a direct action of toxin on higher nervous centers (10). The possibility that endotoxin causes hypotension by action on the heart or on blood vessels generally was investigated in the present series of experiments.

It seems evident that the acute decline in arterial blood pressure, which occurred soon after the injection of endotoxin, was associated with a corresponding decline in cardiac output. There was no significant change or an increase in the calculated peripheral resistance and this suggested that arterial or arteriolar vasodilatation did not occur. The hypotensive state was fully accounted for by a fall in cardiac output to between 21 and 36 per cent of the control value. When cardiac output was artificially maintained at a constant value by a mechanical heart, no significant blood pressure decline was produced by endotoxin, offering further proof that shock was not due to a fall in peripheral resistance.

The inferior vena cava pressure was unaffected or decreased when shock was precipitated, consequently the fall in cardiac output could not be attributed to congestive heart failure. Direct observation of the heart in the open chested dog and electrocardiographic observations supplied no evidence that myocardial dysfunction was a factor in the production of shock.

A definitive demonstration of the hemodynamic changes involved was supplied by the venous return experiments. It was found that the injection of endotoxin produced a marked decrease in venous return. When the blood supply to the right atrium was artificially maintained through an exogenous source, the blood pressure did not fall. The heart was fully capable of normal function and both cardiac output and arterial blood pressure were unaltered as long as the large deficit in venous return was compensated for by augmented blood supply to the right heart.

The immediate hypotensive action of endotoxin in the dog is therefore attributable to an effect upon one or more areas of the body vasculature which results in the pooling of large quantities of blood or the extravasation of fluid. Since peripheral resistance does not fall, pooling must occur predominantly in the post-arteriolar vascular bed. We have already pointed to the portal venous system as a primary site of pooling. The amounts of fluid stored in the liver and intestine, quantitated by a gravimetric method previously described (12), account for most or all of the blood loss which is reflected as deficient venous return in the present experiments. It is to be noted, however, that in the intact animal a fall in the arterial pressure probably limits the extent of rise of liver and intestinal weight. When the blood pressure is maintained at normotensive levels by means of total body perfusion or compensation of deficient venous return, elevation of the portal vein pressure appears to be accentuated and the deficit in venous return is greater.

The initial pooling in the portal system is evidently caused by transient obstruction to venous outflow from the liver. We have measured pressures in unobstructed hepatic veins of dogs following injection of endotoxin and found that these vessels reflect the pressure conditions of the inferior vena cava rather than those of the portal vein. Essex and Thomas (29, 30) have demonstrated occlusive spasm of the intrahepatic vasculature of the dog and cat following administration of *Ascaris suum* extract, hydatid cyst fluid, and histamine, not attributable to a local sphincter mechanism or sluice valve at the junction of the hepatic veins and the inferior vena cava. Endo-

toxins from gram-negative bacteria probably exert a similar action. This is supported by the observation that portal-caval anastomosis (Eck fistula) greatly modifies the immediate hypotensive reaction that follows injection of endotoxin, parasitic extracts, or anaphylaxis (9, 31, 32). However, the hepatovascular phenomena which occur in various types of shock are as yet poorly understood.

There is no evidence at present that pooling occurs in systemic veins in the early period after endotoxin injection. In contrast to the marked elevation of portal vein pressure, a depression in the vena cavae and in small subcutaneous vein pressures was observed after injection of endotoxin.

Evidence that intravenous injection of endotoxin causes a significant increase in capillary permeability is lacking (33) and it is doubtful that leakage of fluid from the intravascular space can be implicated as the major factor in the production of the initial hypotensive state in the dog, although such leakage may be a factor in later stages. Studying shock produced by meningococcal toxin, Ebert, Borden, Hall, and Gold (23) also found a marked fall in cardiac output which was not attributable to changes in peripheral resistance. No regular change occurred in the hematocrit or plasma volume after such shock. However, prolongation of mixing time has been shown recently to be an important limiting factor in the estimation of blood volume during shock (34) and erroneously low volumes may be calculated.

The mechanism of the shock produced by a single injection of endotoxin is in several respects similar to anaphylactic shock and irreversible hemorrhagic shock. Almost a half century ago, Pearce and Eisenbrey experimentally demonstrated deficient venous return as the cause of anaphylactic hypotension by use of a donor dog in place of a reservoir system (35, 36). An increasing body of experimental evidence has led to a re-examination of physiological concepts as to the genesis of shock associated with infections. Although the most readily measured changes occur in the arterial bed, the cause of these changes in the early period after endotoxin has been administered to dogs intravenously is found to be pooling of blood in specific post-capillary vessels.

CONCLUSIONS AND SUMMARY

Hypotension was produced in anesthetized dogs by intravenous injection of endotoxin derived from *Escherichia coli* and *Brucella melitensis*. The sharp fall in arterial pressure was accompanied by a marked rise in portal vein pressure and a fall in systemic venous pressure. Hypotension was produced by the fall in cardiac output without a decrease in the peripheral resistance. No evidence was found to indicate that myocardial failure was a factor in producing the hypotension. Total body perfusion prevented hypotension and intensified portal vein pressure elevation.

Venous return, measured by means of a reservoir system interposed between the great veins and the right heart, decreased markedly after injection of endotoxin. When cardiac filling was kept constant by an infusion system, the deficit in venous return was augmented and the portal vein pressure elevation was intensified, but the arterial pressure did not fall.

These observations indicate that the primary circulatory disturbance produced by endotoxin in dogs is mediated thr ɔugh an action on one or more specialized venous beds, including especially the vasculature of the liver. It is suggested that localized venous spasm in the hepatic venous system and possibly elsewhere produces pooling of large quantities of blood. The total venous return is thereby critically reduced and a fall in cardiac output and arterial blood pressure are inevitable end results, which account for the hypotension in this situation.

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MULTIPLE SERUM PROTEIN DEFICIENCIES IN CONGENITAL AND ACQUIRED AGAMMAGLOBULINEMIA [1]

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The absence of serum γ-globulins in patients afflicted with severe recurring bacterial infections has been reported (1, 2). Three forms of this metabolic disturbance, agammaglobulinemia, have been described (3): 1) a physiologic or transient form occurring in infants, 2) a congenital form which is inherited as a sex-linked recessive characteristic occurring in males, and 3) an acquired form, occurring in both sexes, with the onset of infections in adolescence or adulthood. The congenital and acquired forms of the disease are due to an absence of plasma cells in the tissues of these patients (3–5) with a consequent deficiency of γ-globulin and failure of antibody synthesis (4); the plasma cells appear to be the site of antibody synthesis (6). Unlike the transient form, the failure of γ-globulin synthesis in the congenital and acquired groups, once established, seems to be permanent.

Using rabbit and horse antisera against human γ-globulin in the quantitative precipitin reaction, it had been demonstrated that the failure of γ-globulin synthesis in these patients was usually not complete and that small amounts of γ-globulin were often present in their sera, less than 25 mg. per cent in the congenital form and less than 100 mg. per cent in the acquired form (3). In this report, it will be shown that at least two other plasma proteins in addition to γ-globulin are either absent or markedly deficient in these two forms of the disease.

METHODS

Human sera studied: Sera from 8 children with congenital agammaglobulinemia, from 3 adults with the acquired disease, and from 6 normal children and adults were selected for study. The γ-globulin concentrations in the agammaglobulinemic patients ranged from 0 to 35 mg. per cent as determined immunochemically. All of the sera studied were obtained prior to instituting γ-globulin therapy.

Unadsorbed and adsorbed antisera: 1) Rabbit antiserum against human γ-globulin was prepared as described elsewhere (7). 2) Horse antiserum against human γ-globulin was prepared according to the method described for horse anti-human albumin (8), using as the antigen a purified γ-globulin preparation obtained by low temperature ethanol-water fractionation (9). The horse antiserum gave the diphtheria antitoxin type of precipitation curve in its interaction with human γ-globulin (10). As will be shown, these antisera were *not* entirely specific for γ-globulin alone, since small amounts of antibodies to trace plasma protein components in the γ-globulin fraction used as the antigen were also found. It was the latter "contaminating" antibodies that proved so useful for this study. 3) Horse antiserum prepared by the injection of whole human plasma was kindly supplied by Dr. Robert Berg, of the Massachusetts General Hospital.

Aliquots of 20 ml. of each of the antisera were adsorbed with 3 ml. of a patient's serum by mixing, incubating at 37° C for 1 hour and then at 4° C overnight, and finally centrifuging at 0° C at 3,000 RPM to remove any resulting precipitate. In this manner, aliquots of antisera were adsorbed with sera from 3 children with congenital agammaglobulinemia and additional aliquots adsorbed with sera from 2 normal adults. Adsorption of any given aliquot of antiserum was performed only with the serum from a single individual.

Thus, each of the three antisera was used in 3 forms: 1) unaltered or unadsorbed, 2) adsorbed with agammaglobulinemic serum, and 3) adsorbed with normal serum. There were 3 separate aliquots in the second group corresponding to the three separate agammaglobulinemic sera used for adsorption and similarly there were 2 separate aliquots in the third group. *Each* variation of *each* antiserum was tested against *all* of the normal and agammaglobulinemic sera studied.

Antigen and antibody analysis: A modification of the immunoelectrophoresis method of Grabar and Williams (11) was used for antibody analysis of the antisera and for antigenic analysis of the patients' sera. In this method, the patient's serum was electrophoresed in an agar plate and the plate "developed" by permitting antisera to diffuse horizontally into the agar phase. Interaction of antigen and antibody was indicated by the appearance of a band of specific precipitate in the agar.

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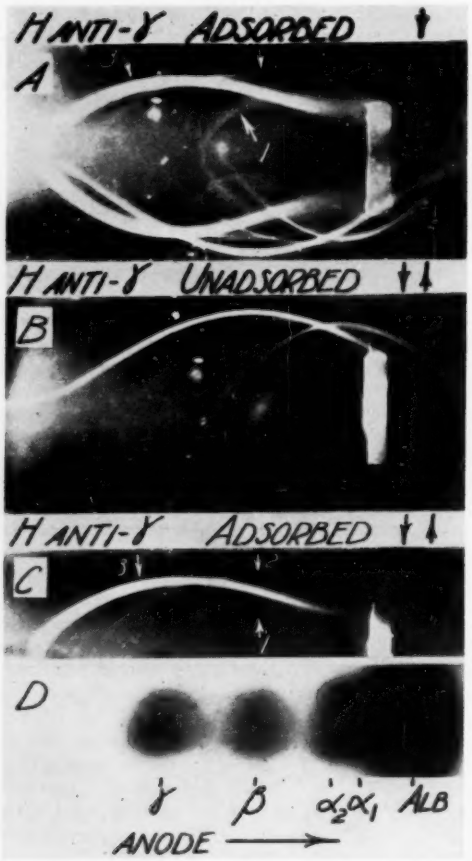

FIG. 1. ELECTROPHORESIS OF NORMAL AND AGAMMAGLOBULINEMIC SERA IN AGAR; DEVELOPED WITH HORSE ANTISERUM AGAINST HUMAN γ-GLOBULIN (H ANTI-γ)

This figure and all subsequent figures represent sections taken from immunoelectrophoresis plates. The horizontal edges indicate the edges of the wells containing antiserum and the vertical white bars on the right half of the figures delineate the transverse wells for the patient's serum-agar solution. The anode was always at the right. Between the agar sections are noted, by arrows, the direction of diffusion of antisera used for development.

A. Electrophoresis of normal serum. Upper portion developed with H anti-γ adsorbed with agammaglobulinemic serum, showing three precipitation bands (white arrows). Lower portion developed with unadsorbed H anti-γ, showing 7 precipitation bands; the eighth band had migrated into the antiserum well by the time this photograph was taken.

B. Electrophoresis of serum from a patient with congenital agammaglobulinemia. Upper portion developed with unadsorbed H anti-γ, showing only 4 of the 7 bands seen in A. Lower part developed with H anti-γ adsorbed with agammaglobulinemic serum; no bands are seen.

C. Electrophoresis of another normal serum developed with the same adsorbed H anti-γ as in 1A and 1B.

D. Electrophoresis in agar of same normal serum as in A but stained with bromphenol blue.

For electrophoresis, pieces of plate glass (¼″ × 8″ × 10″ or ⅛″ × 6″ × 8″) were coated with a thin layer of 1 gm. per cent agar and dried at 100° C. The plates were then edged with plasteline to a height of about one cm.; 3 to 5 bars of lucite, ¼″ × ¾″ × 6″, were arranged in parallel on the plate, dividing its surface into equal longitudinal areas, the bars being placed on the ¼″ edge. Two gm. per cent agar in 0.1 M borate buffer, pH 8.6, was poured over the plate to a depth of 3 to 5 mm. and permitted to gel. The lucite bars and plasteline were removed and narrow linear wells, about 2–3 mm. × 12–20 mm., were cut into the agar transversely to the long dimension of the plate. The patients' sera were dialyzed overnight against 0.2 M pH 8.6 borate buffer and clarified by centrifugation at 24,000 × gravity for 30 minutes; the sera were warmed to 50° C and were then mixed with an equal volume of melted 4 gm. per cent agar, in distilled water, cooled to 50° C. The agar-serum was then placed in the transverse wells and the plate cooled. Electrophoresis was performed at 4° C using 8 to 10 volts per cm. of agar for 16 to 17 hours, using 3 thicknesses of buffer saturated Whatman 3 MM filter paper to make electrical contact between agar plate and buffer reservoirs. After electrophoresis, unadsorbed or adsorbed antisera were placed in the longitudinal wells resulting from removal of the lucite bars and the plates were kept at about 22° C in moist chambers for 1 to 2 weeks; the borate buffer suppressed bacterial and fungal growth during this period of time. Any given antiserum thus diffused laterally into 2 agar-serum sections at once; zones of antigen-antibody interaction were indicated by curvilinear bands of specific precipitate. After the plates were so developed, in some instances, circular wells were cut out of the agar close to a particular band of precipitate and the wells were then filled with 1 gm. per cent γ-globulin or other plasma protein fractions; similarity or identity between the antigen in the patient's serum and that in the circular well was indicated by a shift in the position of the precipitation band. All plasma protein fractions used in this study were isolated by low temperature ethanol-water fractionation (9, 12).

In some instances, the agar plates were stained with bromphenol blue, instead of being developed with antisera, to determine the extent of migration of the plasma proteins under the electrophoretic conditions employed.

It should be noted that separation of the α-globulins in borate buffer is less satisfactory than with barbiturate buffer, but that separation between the γ and β-globulins is relatively greater than that obtained with barbiturate buffer (Figure 1D).

RESULTS

Analysis with horse antiserum vs. human γ-globulin

The unadsorbed horse antiserum against human γ-globulin (H anti-γ) resulted in the appearance of 8 precipitation bands when reacted with normal human sera (Figure 1A). When adsorbed with any of three sera from children with agammaglobulinemia and then again reacted with normal human serum, only 3 bands were obtained (Figures 1A and 1C). Thus, agammaglobulinemic sera failed to remove 3 antibodies against normal serum proteins from H anti-γ. In each instance, one of these 3 remaining precipitation bands (arrow 3) could be demonstrated to be due to the interaction of γ-globulin with its homologous antibody (Figure 4); the other two bands (arrows 1 and 2) were attributable to the interaction of two β-globulins with their respective antibodies. It should be noted that in no instance did the *adsorbed* antisera give any visible reaction against any of the sera from patients with congenital (Figure 1B) or acquired agammaglobulinemia. In view of this evidence of completeness of adsorption of antibodies against *agammaglobulinemic sera,* the 3 antibodies remaining against normal serum proteins indicated the absence of 3 corresponding serum proteins from agammaglobulinemic sera. Adsorption of the antiserum with normal human serum removed all precipitating antibodies for both agammaglobulinemic and normal sera.

When reacted with agammaglobulinemic sera, unadsorbed H anti-γ gave 5 bands in the agar plate (Figure 1B). Thus, there were 3 fewer bands in each instance than when the same antiserum reacted with normal serum. The missing 3 bands corresponded to the 3 bands found when reacting agammaglobulinemia-adsorbed antiserum with normal sera.

Analysis with rabbit antiserum vs. human γ-globulin

The unadsorbed rabbit antiserum against human γ-globulin (R anti-γ) showed 5 bands when reacted with normal human sera (Figure 2A). By far the most dense band was that due to γ-globulin reacting with its homologous antibody. The remaining bands of specific precipitate were exceedingly faint and difficult to detect. When reacted with agammaglobulinemic sera, it was noted that, but for one case, R anti-γ still gave 5 bands. In these instances, however, the γ-globulin band was much fainter and broader (arrows, Figure 2B)

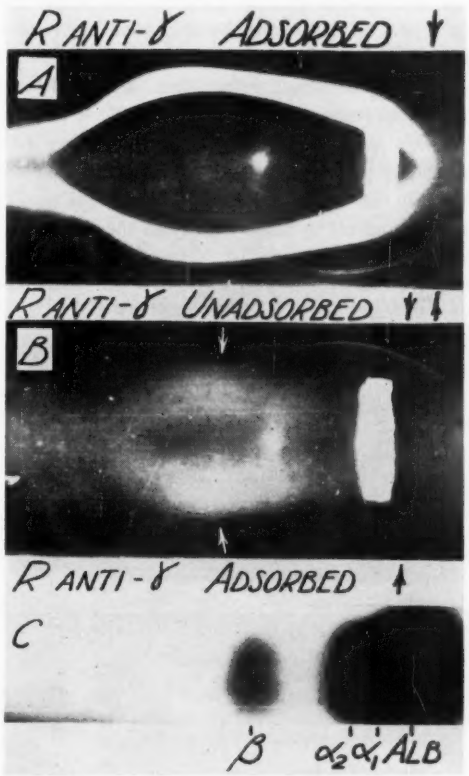

FIG. 2. AGAR PLATES DEVELOPED WITH RABBIT ANTISERUM AGAINST HUMAN γ-GLOBULIN (R ANTI-γ)

A. Electrophoresis of a normal serum. Upper part, developed with R anti-γ adsorbed with the agammaglobulinemic serum used in 2B, shows only the single band due to interaction of human γ-globulin with specific antibodies. Lower part developed with unadsorbed R anti-γ; 4 bands can be discerned in this photograph.

B. Electrophoresis of serum from a patient with congenital agammaglobulinemia. Upper part, developed with unadsorbed R anti-γ and lower part developed with the same adsorbed R anti-γ used in 2A. Diffuse bands (white arrows) were due to presence of small amount of γ-globulin in this patient's serum. Although this serum was used for adsorbing R anti-γ, the amount of γ-globulin present was insufficient to remove all anti-γ globulin antibodies.

C. Electrophoresis of same serum as in 2B, but stained with bromphenol blue; no γ-globulin can be discerned in the serum with this method.

and its position toward the center of the longitudinal axis of the agar plate clearly indicated a markedly reduced concentration of γ-globulin. In the exception noted, no γ-globulin band could be detected with R anti-γ; this serum came from a patient with acquired agammaglobulinemia and contained no γ-globulin by the quantitative precipitin method.

When rabbit antiserum was adsorbed with agammaglobulinemic serum, only the γ-globulin antibody remained in the antiserum (Figure 2A). Although γ-globulin was detected in all but one of the agammaglobulinemic sera tested, the amount was insufficient to remove much of the rabbit antibodies against γ-globulin when these sera were used for adsorption. Adsorption with normal serum removed all detectable antibodies from R anti-γ.

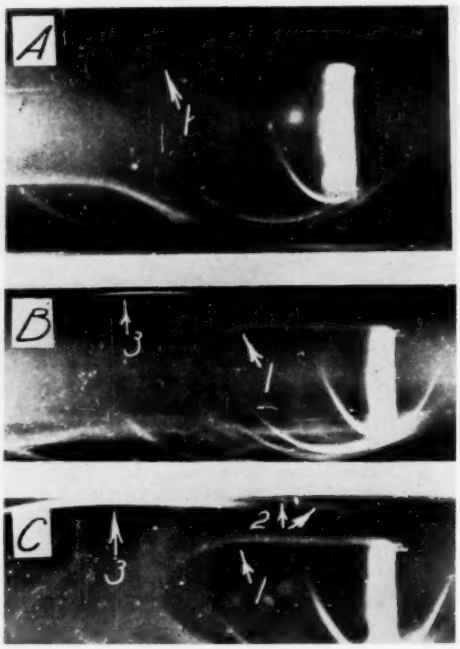

FIG. 3. ELECTROPHORESIS OF A NORMAL SERUM IN AGAR

Only the upper portions of each section will be referred to.

A. Developed with horse antiserum against whole human plasma adsorbed with serum from a patient with congenital agammaglobulinemia. After 5 days only the band indicated by arrow 1 could be seen.

B. The antiserum was then replaced with similarly adsorbed H anti-γ. A day later, band 3 was noted.

C. Three days later band 2 was apparent.

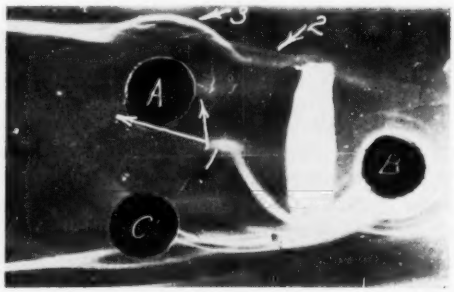

FIG. 4. ELECTROPHORESIS OF A NORMAL SERUM IN AGAR, DEVELOPED WITH UNADSORBED H ANTI-γ (LOWER PORTION) AND AGAMMAGLOBULINEMIA-ADSORBED H ANTI-γ (UPPER PORTION)

The circular wells A, B and C were then cut into the agar and the wells filled with various plasma protein fractions:

A. β-isohemagglutinin
B. Impure α-lipoprotein
C. Iron-binding globulin

The resolution and reprecipitation of the γ-globulin band to form a semicircle at arrow 3 indicated the presence of γ-globulin in the β-isohemagglutinin fraction used. The 2 β-globulin precipitation bands remained unaffected by the isohemagglutinin.

Analysis with horse antiserum vs. whole human plasma

With horse anti-whole human plasma, 8 bands were observed when reacted with normal sera and 6 bands with agammaglobulinemic sera. The human γ-globulin precipitation band, when present, was exceedingly faint in this instance. Adsorption with agammaglobulinemic sera resulted in the elimination of all but one band; this proved to be the same as one of the β-globulin bands observed with H anti-γ (Figure 3, arrow 1). Since the agammaglobulinemic sera used for adsorption contained some γ-globulin and since the concentration of antibodies against γ-globulin in this antiserum was very low, adsorption completely removed these antibodies. The β-globulin band, however, could be better visualized with this antiserum than with H anti-γ. In one instance, there was a question as to whether some of the antigen causing this band was also present in diminished amount in a single patient with congenital agam-

maglobulinemia. By developing a plate first with agammaglobulinemia-adsorbed horse anti-whole plasma for about a week and following this with agammaglobulinemia-adsorbed H anti-γ, all three of the bands due to the antigens missing or diminished in agammaglobulinemic sera could be visualized (Figures 3B and 3C, arrows). Adsorption with normal sera eliminated all precipitation bands.

Attempts at identification of the β-globulins

It is known that isohemagglutinins are absent or deficient in the two forms of agammaglobulinemia studied here (2, 3). Neither of the two β-globulins in question, however, appeared to be isohemagglutinins. Adsorption of the horse antisera with isohemagglutinins isolated from normal plasma did not remove the antibodies for these β-globulins. In addition, diffusion of isohemagglutinins into the agar plates already developed with antisera failed to shift the precipitation bands associated with the unknown β-globulins (Figure 4). Although other plasma protein fractions, such as α_1- and α_2-glycoproteins, α- and β-lipoproteins, and iron-binding globulin, were similarly studied, the β-globulins could not be identified. From the results, it is clear, however, that they have no antigenic relationship with γ-globulin.

DISCUSSION

It would appear from the results presented that at least two plasma proteins besides γ-globulin are absent or markedly diminished in congenital and acquired agammaglobulinemia. These proteins migrate as β-globulins and have no apparent antigenic relationship to γ-globulin. Since the individual components of γ-globulin are immunochemically related and cross-react with antisera against γ-globulin, the data clearly indicate that these two β-globulins cannot be considered as fast-moving components of γ-globulin. The failure of synthesis of γ-globulin in these patients is presumably due to the absence of plasma cells; it is interesting to speculate whether these β-globulins are also products of the plasma cell. However, further study is necessary to establish the identity and function of these proteins. It would appear, from the intensity of the reaction and the position of the precipitation bands in the electrophoresis plates, that the concentration of at least one of these proteins, indicated by arrow 1 in Figures 1 and 3, even in normal plasma is not very great. This does *not*, of course, preclude its possible physiological importance. It must be emphasized that there may be *additional* plasma proteins absent or deficient in this syndrome; the number of proteins which could be studied in this investigation depended entirely upon the number of antibodies present in the antisera used.

It has been shown that the deficiency of γ-globulin in 10 of the sera of the 11 patients with agammaglobulinemia studied was not complete, as has already been pointed out (2, 3); in one instance, no precipitation band for γ-globulin could be obtained even with the rabbit antiserum. The reasons for the apparently greater sensitivity of rabbit antiserum than horse antiserum in the detection of human γ-globulin lies in the differences in the precipitation characteristics of the two types of sera, the horse antiserum requiring a greater amount of γ-globulin for visible precipitation than does the rabbit antiserum under similar conditions (10).

SUMMARY

1. Antigenic analysis of sera from 8 patients with congenital agammaglobulinemia, 3 patients with the acquired form of the disease and 6 normal adults was performed, using rabbit and horse antisera. The rabbit and horse antisera against human γ-globulin contained antibodies against other plasma antigens besides γ-globulin.

2. Through use of agar diffusion and electrophoretic methods, it has been demonstrated that at least 2 plasma proteins besides γ-globulin are absent or deficient in agammaglobulinemia; these proteins migrate as β-globulins in agar.

3. It is confirmed that the deficiency of γ-globulins in agammaglobulinemia is often *not* complete.

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OXYGEN CONSUMPTION IN DOGS AS INFLUENCED BY THE ALTERED METABOLISM OF DIABETES MELLITUS

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(Submitted for publication December 12, 1955; accepted July 18, 1956)

The question of the total energy production in the human diabetic has been studied extensively by Benedict and Joslin (1, 2) and by Allen and Du Bois (3). It generally has been agreed that energy metabolism in mild cases of diabetes is normal, and that in the more severe cases is found increased when determined on the basis of kilogram of body weight. The lack of a satisfactory standard of comparison between diabetic and nondiabetic subjects hampered much of the earlier work in this field and led to disagreement regarding interpretation of data. Benedict and Joslin (1, 2), using the Harris-Benedict prediction standard, reported increases in metabolic rate of diabetic subjects of between 15 and 20 per cent. Lusk (4) criticized these data and computed that the increase in metabolism did not exceed 5 per cent. With the general acceptance of the more valid Du Bois standard (5) comprehending body surface area, many of the earlier data ascribing an increase in metabolic rate to diabetes have been recalculated and shown to be erroneous. More recent publications likewise have been of conflicting nature reporting increases in metabolic rate as great as 40 per cent and decreases of 32 per cent in severe clinical diabetes mellitus (6–9).

In the pancreatectomized dog, on the other hand, respiratory metabolism generally has been found to be increased (10–12). However, attempts at direct comparison between this type of acute canine diabetes and the disease as it occurs in humans is illogical in view of the typical alteration in fatty acid metabolism following pancreatectomy. We are not aware that oxidative metabolism has been measured previously in the alloxan diabetic animal which provides a metabolic defect more nearly identical with that occurring in human diabetes mellitus.

That an increase in oxygen consumption in diabetes mellitus probably does not result from a uniform acceleration of metabolism of all body tissues, has been supported by the work of Kety, Polis, Nadler, and Schmidt (13) and Schecter, Wiesel, and Cohn (14) who demonstrated a decrease in oxygen utilization by the brain and extremities in patients with diabetic acidosis. In this report the basal total body oxygen consumption has been determined for the diabetic dog and a study made of its modification by insulin administration or hypophysectomy. Furthermore, the influence of experimental diabetes mellitus upon the hepatic blood flow and splanchnic oxygen consumption has been determined.

MATERIAL AND METHODS

Adult female dogs were used ranging in weight from 8 to 20 Kg. and housed indoors in a relatively constant environmental temperature of 24 to 26° C. All animals were fed a fixed daily ration consisting of 10 gm. of Purina Laboratory Chow pellets and 10 gm. of horsemeat per kilogram. Five of the pancreatectomized animals received 100 gm. of fresh beef pancreas daily for 20-day periods which was substituted in the diet during that time for an equivalent quantity of horsemeat. Water was offered *ad libitum*.

The rate of oxygen consumption [2] of the entire animal was obtained by an open circuit method previously described in detail (15) utilizing Liston-Becker and Beckman carbon dioxide and oxygen analyzers in the expiratory system to provide a constant index of the composition of the expired air. When the animal was in a basal state after a period of at least 30 minutes of complete rest, two successive samples of the dried expired air were drawn from the system in duplicate at an interval of 10 minutes and analyzed by the method described by Scholander (16). Provided the oxygen analyses of the two samples agreed within 0.05 per cent, the determination was believed to be reliable and oxygen consumption of the dog was calculated. The data have been converted to their respective values for standard conditions of temperature and barometric pressure.

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[2] The oxygen consumption apparatus was designed and constructed by Captain F. A. Miller.

All determinations of oxygen consumption were carried out on the trained unanesthetized animal excepting those done in conjunction with the estimation of hepatic blood flow and splanchnic oxygen consumption. These latter experimental procedures were performed under sodium pentobarbital anesthesia. Care was taken with the training of the unanesthetized dogs in order to ensure the maintenance of a basal condition. All animals were studied 24 hours after the last ingestion of food and at complete rest, being devoid of all voluntary or extraneous involuntary movement. Individual animals were studied repeatedly under control and experimental conditions until a plateau level for oxygen consumption was reached which could be reproduced from day to day. Under these circumstances the lowest rates of oxygen utilization were incorporated into the data. Basal oxygen consumption determinations in the pancreatectomized dogs were not believed to be totally reliable until after the incision was well healed, a period of approximately two weeks. Rectal temperature for each dog was measured immediately following an oxygen consumption determination. Only animals whose temperature fell within the range $38 \pm 0.5°$ C were acceptable for this series. The basal per kg. oxygen consumptions of 51 well trained normal dogs determined by this procedure have been found to be in good general agreement with similarly acquired values obtained by other laboratories (17).

The bromsulphthalein (BSP) extraction method for the estimation of hepatic blood flow as described by Bradley, Ingelfinger, Bradley, and Curry (18) was applied to dogs in order to provide a means whereby the splanchnic oxygen consumption might be determined in diabetes mellitus. This technique has been compared in the dog with simultaneous direct measurement of hepatic blood flow by Selkurt (19) who reports values for the BSP method exceeding direct measurement by approximately 7 per cent. This difference is thought to be due to a small extrahepatic loss of the dye. Under sodium pentobarbital anesthesia (25 mg. per Kg.) and using fluoroscopic control a number 6 or 8 Cournand type cardiac catheter was inserted via the external jugular vein into a large hepatic vein in either the right or left lobe of the liver. Indwelling polyethylene cannulae were inserted high into the external saphenous vein and into the right atrium through the external jugular vein. After a priming dose of 50 to 100 mg. of BSP, an infusion of approximately 2.0 mg. BSP in isotonic saline per minute was delivered through the intra-atrial cannula by a calibrated constant infusion apparatus. A slow infusion of isotonic sodium chloride was passed through the hepatic and saphenous cannulae to maintain their patency when not in use. Following a 30-minute period to allow for stabilization of the peripheral BSP level, three pairs of blood samples were drawn at 10-minute intervals simultaneously from the hepatic and saphenous veins for BSP analysis. Hepatic venous and femoral arterial bloods for determination of oxygen content were obtained just prior to the second BSP sampling. Plasma BSP levels were measured colorimetrically as described by Myers (20) and blood oxygen content was measured by the manometric method of Peters and Van Slyke (21). Hepatic blood flow was calculated as outlined by Bradley, Ingelfinger, Bradley, and Curry (18) and the splanchnic oxygen consumption was derived from the product of the hepatic blood flow and the arterial-hepatic venous oxygen difference. The maximum observed rate of change in concentration of the peripheral BSP was .0005 mg. per ml. per minute. The total blood volume was assumed to be 100 ml. per Kg. of body weight, this value was derived from the data of Krieger, Storaasli, Friedell, and Holden (22). All experiments were carried out at $38 \pm 0.5°$ C body temperature.

The subtemporal intracranial approach was utilized for hypophysectomy as has been described previously by Keller, Lawrence, and Blair (23). Pancreatectomy was performed through a longitudinal mid-line incision, taking care to remove all pancreatic tissue. Alloxan diabetes was induced by the rapid intravenous administration of 75 to 100 mg. per Kg. of alloxan. Phlorhizin diabetes was produced in the fasted dog by the subcutaneous administration of 2 gm. of phlorhizin suspended in olive oil on two successive days with the chemical and metabolic determinations in these animals being made 24 hours following the last dose of the drug. Recovery from

TABLE I

INFLUENCE OF ACIDOSIS, KETOSIS AND DIABETES MELLITUS UPON OXYGEN CONSUMPTION IN DOGS

	NUMBER OF DOGS	WEIGHT Kg.	OXYGEN CONSUMPTION ml./kg./min.	RQ	BLOOD SUGAR mg./100 ml.	BLOOD ACETONE mg./100 ml.	BLOOD TOTAL KETONE mg./100 ml.	pH	PLASMA CO_2 CONTENT mEq./L.
CONTROL	6	11.4 ± 1.0†	5.89 ± .65	.75 ± .10	82 ± 10	0.9 ± 0.3	2.2 ± 0.8	7.36 ± .02	23.6 ± 1.4
ACIDOSIS	6	11.3 ± 1.1	5.96 ± .95	.73 ± .04	85 ± 9	1.0 ± 0.4	1.6 ± 0.5	7.10 ± .04*	9.9 ± 2.1*
FASTING	6	10.1 ± 1.0*	5.87 ± .40	.64 ± .09	65 ± 9*	1.9 ± 0.2	4.6 ± 1.7*	7.32 ± .06	22.7 ± 2.4
PHLORHIZIN DIABETES	6	9.9 ± 1.1*	7.66 ± .74*	.70 ± .04	41 ± 8*	11.5 ± 2.8*	20.9 ± 7.7*	7.22 ± .10*	16.8 ± 2.4*
ALLOXAN DIABETES	3	9.5 ± 0.4*	7.82 ± .57*	.68 ± .02	635 ± 187*	3.1 ± 1.6*	5.5 ± 2.4	7.22 ± .04*	17.2 ± 2.4*
PANCREATECTOMY DIABETES	3	9.3 ± 0.4*	8.86 ± .08*	.66 ± .03	228 ± 53*	9.7 ± 3.0*	22.7 ± 6.9*	7.19 ± .05*	16.7 ± 3.4*

† MEAN ± STANDARD DEVIATION.
* DENOTES A SIGNIFICANT DIFFERENCE FROM THE MEAN OF THE CONTROL SERIES AT THE $P < .05$ LEVEL, FROM FISHER'S TABLE OF T VALUES.

TABLE II

INFLUENCE OF INSULIN UPON THE OXYGEN CONSUMPTION OF ALLOXAN AND PANCREATECTOMIZED DIABETIC DOGS

		OXYGEN CONSUMPTION ml./Kg./min.	RQ	BLOOD SUGAR mg./100ml.	URINE SUGAR gm./Kg./day	BLOOD ACETONE mg./100ml.	BLOOD KETONE BODIES mg./100ml.	pH	PLASMA CO_2 CONTENT mEq./liter	WEIGHT KILOGRAMS	PROTAMINE ZN INSULIN units/day
UNCONTROLLED DIABETES	PANCREATECTOMY	8.65+ (8.03-8.96)*	.67 (.60-.72)	253 (173-308)	4.01 (1.28-6.32)	11.0 (6.7-15.4)	24.8 (12.1-32.1)	7.18 (7.08-7.25)	17.7 (14.2-23.9)	9.5 (9.0-10.0)	0 0
	ALLOXAN	7.72 (7.37-8.22)	.68 (.62-.68)	192 (142-251)	5.39 (4.26-6.05)	1.7 (0.9-2.7)	4.5 (2.2-7.6)	7.32 (7.26-7.38)	23.0 (22.2-24.6)	10.8 (9.0-14.1)	0 0
INSULIN CONTROLLED DIABETES	PANCREATECTOMY	7.95 (7.51-8.36)	.67 (.66-.70)	87 (60-106)	0.62 (.04-1.27)	1.4 (0.6-2.3)	3.8 (3.4-4.0)	7.31 (7.28-7.37)	21.7 (20.9-22.3)	9.4 (9.2-9.8)	6 (5-7)
	ALLOXAN	5.82 (5.32-6.39)	.70 (.66-.73)	74 (37-128)	1.39 (.33-2.71)	1.2 (0.9-1.5)	2.8 (1.5-4.8)	7.37 (7.35-7.38)	22.5 (22.3-22.8)	11.1 (9.0-14.4)	9 (7-10)

+ MEAN
* RANGE

the effects of phlorhizin was considered complete with return of the fasting blood sugar to the range of 70 to 90 mg. per 100 ml. and the disappearance of glucosuria. Further use of phlorhizin poisoned animals was delayed until these criteria were satisfied. In view of the increased susceptibility of the diabetic dog to infection, sufficient penicillin was administered during the course of the experiment to prevent the development of any septic condition. At termination the brain in the hypophysectomized dogs was removed with the fossal scar remaining undisturbed and still attached to the ventral part of the hypothalamus. After fixation in Bouin's solution, the blocks of the hypophysis-hypothalamus were sectioned sagittally and serially. Appropriate sections were mounted and stained by Maximow, Pal-Weigert, Mallory, and Heidenhain procedures. The anatomical degree of hypophysectomy was estimated from the histological sections and classified as defined by Keller, Lawrence, and Blair (23).

Fasting blood sugar, 24-hour urine sugar output, arterial acetone and total ketone bodies (24), arterial pH and arterial plasma carbon dioxide content determinations were performed immediately following oxygen consumption procedures. The analysis of total liver lipids was carried out on both biopsy and autopsy liver specimens as described by Kaplan and Chaikoff (25). Biopsy wedges of liver tissue weighing approximately 3 gm. were obtained under general anesthesia the day following oxygen consumption determinations. Insulin tolerance to a given dose of crystalline insulin was ascertained for each hypophysectomized dog prior to pancreatectomy to serve as a physiological index of the completeness of hypophysectomy as reflected by the extent of the available carbohydrate reserve (23).

RESULTS

The data comprising Table I show that acidosis resulting from the ingestion of ammonium chloride (0.4 gm. per Kg. three times daily for two days) did not alter materially the oxygen consumption of 6 dogs from that of the control period. The mean arterial pH of 7.10 and carbon dioxide content in the plasma of 9.9 mEq. per liter indicate an acidosis of comparable degree to that of diabetic acidosis. It is shown also that a fasting period of 10 days with a resultant average weight loss of 11 per cent produced no change in oxygen consumption. In contrast to the lack of effect of these two factors, the injection of phlorhizin into each of the 6 dogs, while maintained under fasting conditions, caused an increased oxygen utilization exceeding 20 per cent in 5 of the 6 animals. This observation has been reported by Lusk (26) and confirmed by Boothby, Wilhelmj, and Wilson (27), although its mechanism has not been determined. In 3 dogs the basal oxygen consumption following establishment of the diabetes by pancreatectomy exceeded the control for these animals by approximately 45 per cent. Of the 3 alloxan diabetic dogs, all exhibited increases in metabolic rate following the production of the diabetes. One of the 3 dogs' metabolic rate was elevated to the same general degree as that of the pancreatectomized dogs; in the remaining 2 the increases were 18 and 29 per

TABLE III

OXYGEN CONSUMPTION IN HYPOPHYSECTOMIZED DIABETIC DOGS

DOG NUMBER	DATE	LESION	INSULIN TOLERANCE UNITS/Kg.	HOUSSAY EFFECT	WEIGHT Kgs.	OXYGEN CONSUMPTION ml./Kg./min.	BLOOD SUGAR mg./100 ml.	BLOOD ACETONE mg./100 ml.	BLOOD TOTAL KETONE BODIES mg./100 ml.	pH	PLASMA CO_2 CONTENT mEq./L.	URINE SUGAR gm./Kg./DAY	PROTAMINE ZINC INSULIN UNITS/DAY
347	16 SEP 1953	ORDINARY HYPOPHYSECTOMY			15.2								
	19 MAR 1954		1/10	TRACE	12.5	5.67	61	0.9	1.8	7.32	22.7	0	0
	23 MAR 1954	PANCREATECTOMY			12.3								
	6 APR 1954				10.3	6.60	374	2.5	4.5	7.22	22.0	0.82	0
	15 APR 1954				9.4	8.53	466	10.0	17.0	7.25	16.2	1.96	0
345	11 MAY 1953	TOTAL HYPOPHYSECTOMY PLUS MODERATE INFRINGEMENT ON HYPOTHALAMUS											
	14 JUL 1954		1/40	MODERATE	16.0	3.62	49	1.6	1.7	7.33		0	0
	20 SEP 1954	PANCREATECTOMY			16.6								
	5 OCT 1954				14.7	3.86	95	0.9	2.3	7.37	21.3	.04	1
	14 OCT 1954				14.7	5.58	305	0.6	3.6	7.24	26.5	1.05	0
295	25 FEB 1954				16.2	6.64							
	6 APR 1954	HYPOPHYSIAL STALK SECTION WITH CONSIDERABLE INFRINGEMENT ON PARS ANTERIOR		TRACE									
	9 SEP 1954		1		15.1	5.48	79	0.7	1.2	7.38	24.0	0	0
	8 NOV 1954	PANCREATECTOMY			16.0								
	15 NOV 1954				14.0	8.04	252	8.8	15.4	7.41	21.9	2.61	0
	2 DEC 1954				12.5	5.76	70	1.0	1.5	7.31	19.5	.55	4
342	18 AUG 1953	NEAR TOTAL HYPOPHYSECTOMY			9.4								
	21 MAY 1954		1/40	COMPLETE	12.3	4.57	55	1.1	1.5	7.21	24.8	0	0
	1 DEC 1954	PANCREATECTOMY			13.3								
	14 DEC 1954				11.2	6.93	83	2.1	7.0	7.26	20.0	0	0
	29 DEC 1954				10.7	5.28	25	0.3	3.1	7.26	19.4	0	0
343	19 AUG 1953	ORDINARY - PLUS HYPOPHYSECTOMY			8.7								
	6 MAY 1954		1/40	MARKED	11.0	3.88	77	0.9	1.3	7.34	25.1	0	0
	1 DEC 1954	PANCREATECTOMY			10.0								
	17 JAN 1955				7.9	5.99	72	2.9	2.9	7.30	18.4	.53	0
	3 FEB 1955				7.8	8.02	185	2.9	3.6	7.32	20.5	.94	0
406	27 SEP 1954				10.0	5.04	55	0.7	1.0	7.39	22.9	0	0
	5 OCT 1954	500 mg. ALLOXAN			10.0								
	19 NOV 1954				9.3	7.37	182	2.7	7.6	7.26	22.3	4.26	0
	29 NOV 1954	NEAR-TOTAL HYPOPHYSECTOMY		MARKED									
	14 DEC 1954		1/40		9.0	4.27	73	1.6	1.9	7.31	22.0	.62	0
	18 JAN 1955				8.6	4.70	59	1.5	2.1	7.25	21.7	.34	0

cent. At no time during the diabetic period did the oxygen consumption approach control levels in any animal provided insulin was withheld and the diabetes went uncontrolled.

Since increased rates of oxygen consumption generally were measured in association with elevated ketone body concentrations of the peripheral blood, the blood ketone concentration was artificially elevated in two normal dogs. Acetone and beta-hydroxybutyric acid were injected intravenously using 90 gm. of acetone and 150 mg. of beta-hydroxybutyric acid per kilogram of body weight to produce in one hour after administration elevations in circulating ketone bodies of the magnitude found in diabetic acidosis. At that time the rate of oxygen consumption was found to be normal; this tended to eliminate the presence of ketonemia *per se* as cause for the previously observed elevated rates of oxygen consumption in diabetes mellitus.

The influence of protamine zinc insulin upon the oxygen consumption and carbohydrate metabolism in alloxan and pancreatectomized diabetic dogs is demonstrated by the data in Table II. The insulin requirement for suppression of glucosuria, maintenance of the fasting blood sugar at about 100 mg. per 100 ml., and prevention of the acidosis and ketonemia was similar for both groups. The data of Table II express the arithmetic mean and the range of values obtained for the 3 dogs comprising each group prior to and following insulin administration. The oxygen utilization, during treatment with insulin, uniformly decreased in all of the dogs, although the magnitude of reduction was only 10 per cent in the pancreatectomized animals whereas it reverted to control levels in dogs made diabetic with alloxan. The data indicate that the increased oxygen consumption observed in the pancreatectomized dog is brought about by multiple factors, only one of which is the absence of insulin. That incident to alloxan diabetes apparently results solely from insulin deficiency.

In Table III the data illustrate how the amelioration of diabetes mellitus by hypophysectomy influences the rate of oxygen consumption. As will be noted from the Table the anatomical degree of hypophysectomy varied from animal to animal as did their carbohydrate reserve as measured by the insulin tolerance. The magnitude of the ensuing Houssay effect likewise was found to be variable.

TABLE IV

METABOLIC EFFECTS OF EXPERIMENTAL DIABETES MELLITUS IN THE DOG.

	NUMBER OF DOGS	HEPATIC BLOOD FLOW ml./Kg./min.	SPLANCHNIC OXYGEN CONSUMPTION ml./Kg./min.	TOTAL OXYGEN CONSUMPTION ml./Kg./min.	BLOOD SUGAR mg./100 ml.	BLOOD ACETONE mg./100 ml.	BLOOD TOTAL KETONES mg./100 ml.	pH	PLASMA CO_2 CONTENT mEq./L.	ARTERIAL-HEPATIC VENOUS OXYGEN DIFFERENCE vol. per cent	URINE SUGAR gm./Kg./day	TOTAL LIVER LIPIDS per cent	PZI units/day
CONTROL	5	37.3± 9.8*	2.00± .47	6.68± .72	77± 7	1.9± .5	3.0± .9	7.33± .01	23.0± 1.8	5.69± 2.02	-	-	0
PHLORHIZIN DIABETES	5	61.9±83.1*	4.45±1.15*	7.83± .43*	52± 8*	9.4±1.3*	16.8±5.1*	7.23± .02*	20.2± 1.8	7.91± 2.71	-	-	0
CONTROL	5	42.2± 8.9	1.70± .44	6.02± .42	74± 2	1.3± .3	2.0± 1.1	7.36± .01	23.0± 1.5	4.28± 1.49	-	-	0
PANCREATECTOMY DIABETES	5	95.5±29.8*	4.90± .90*	9.23± .43*	312±36*	23.1±3.3*	43.9±4.1*	7.12± .12*	13.8± 4.4*	5.66± 2.58	3.80±.88	25.6±4.5	0
CONTROL	5	35.6± 5.9	1.98± .47	6.85± .45	74± 6	1.8± .4	2.9± 1.0	7.34± .02	22.5± 1.2	5.82± 1.96	-	-	0
PANCREATECTOMY DIABETES WITH INSULIN	5	71.0±33.6*	3.50±1.56	7.51±1.29	83±34	1.9± .4	3.0± .7	7.30± .01*	24.5± 1.9	4.90± .60	1.16±.77	10.0±7.8	2.4
CONTROL	5	36.1± 13.4	1.97± .47	6.09± .44	74± 7	1.8± .4	2.8± 1.0	7.34± .01	22.0± 1.2	5.76± 2.20	-	-	0
PANCREATECTOMY DIABETES WITH INSULIN AND PANCREAS	5	75.8±29.5	4.06±1.52*	7.15± .65*	106±39	2.1± 1.5	4.2±2.5	7.28± .02*	22.5± 2.0	5.03± .34	0.66±.30	9.0±6.2	20.5
CONTROL	5	42.2± 9.0	1.70± .44	6.02± .39	74± 2	1.3± .3	3.1± 1.0	7.36± .01	23.0± 1.5	4.28± 1.49	-	-	0
PANCREATECTOMY DIABETES WITH INSULIN AND CHOLINE	5	42.4±15.9	2.77± .88	6.85±1.63	73±16	1.7± .3	2.8± .5	7.30± .02*	23.8± 2.4	6.78± 1.92*	0.52±.33	6.8±0.2	21.5

* MEAN ± STANDARD DEVIATION.
● REPRESENTS A SIGNIFICANT DIFFERENCE FROM THE CONTROL SERIES ($P<0.05$) FROM FISHER'S TABLE OF t VALUES.

Nevertheless, all 6 pancreatized hypophysectomized animals exhibited an attenuated diabetes mellitus (Houssay effect) as evidenced by a relatively mild hyperglycemia, glucosuria, ketosis, acidosis and intermittent spontaneous episodes of hypoglycemia. The rate of oxygen consumption of the Houssay dog varied considerably from day to day. The lower rates coincided with periods of remission of the diabetes and did not exceed those of the normal dog, whereas maximal rates of oxygen consumption were associated with periods of exacerbation of the diabetes and approached or exceeded 8.0 ml. per kilogram per minute in 3 of the experimental animals. The mean hepatic lipid of the 6 Houssay dogs as analyzed chemically at termination was 11.9 per cent. This figure represents a mild fatty infiltration of the liver in contrast to the 30 to 50 per cent lipid level reported following pancreatectomy (25).

The determination of the splanchnic bed oxygen uptakes for groups of 5 pancreatectomized dogs and their rates of total body oxygen consumption appear in Table IV. These data are compared with control measurements prior to diabetes. Phlorhizin diabetes or simple pancreatectomy diabetes results in significantly increased rates of total body oxygen consumption, hepatic blood flow and splanchnic oxygen consumption. Whereas under control conditions the mean splanchnic oxygen uptake represented approximately 30 per cent of the total body oxygen consumption, this ratio exceeded 50 per cent following injection of phlorhizin or pancreatectomy. This disproportionate increase in rate of liver metabolism in diabetes mellitus was of sufficient magnitude to account for the absolute elevation measured in total oxygen utilization. The administration of insulin to pancreatectomized dogs for periods of 3 weeks, either alone or in combination with lipotropically active substances by mouth (fresh beef pancreas 100 gm. per day or choline chloride 2 gm. per day) was found to prevent a measurable increase in oxygen utilization in approximately 40 per cent of the animals. The combination of insulin with choline was found to be most efficacious in this respect since the mean rate of hepatic blood flow was unchanged and increases in oxygen consumption were small and insignificant in choline fed dogs following pancreatectomy. The lipid content of liver biopsies could not be correlated with the rate of splanchnic oxygen consumption. Massive infiltration of the liver with fat, however, was associated with consistently elevated rates of splanchnic bed metabolism, but similar increases frequently were observed with only minor increments in the level of total liver lipids.

DISCUSSION

In view of the importance of fatty acids as an energy source in uncontrolled diabetes mellitus, their incomplete oxidation and excretion in the urine as ketone bodies might be expected to result in a poor economy of combustion leading to an increased oxygen utilization under "basal" conditions. Since the partial preliminary oxidation of fats is primarily a function of the liver, attention has been focused upon this organ as a possible site of increased metabolic activity in diabetes mellitus which might be in large part responsible for the measured increase in total body oxygen consumption. Therefore the splanchnic oxygen uptake in the diabetic dog has been determined and used as a measure of rate of liver metabolism.

The foregoing experimental procedures have indicated that the metabolic condition giving rise to a state of ketosis whether resulting from diabetes mellitus of phlorhizin, alloxan, or pancreatectomy etiology nearly always is accompanied by increased rates of oxygen consumption. The usual reduction in respiratory quotient accompanying diabetes mellitus necessarily would reduce the caloric equivalent for utilized oxygen to a slight degree (3 per cent) in certain of the diabetic preparations. However, the observed changes when present were of such magnitude as to eliminate this as a factor of importance. The administration of insulin to or hypophysectomy of the diabetic dog have been found to reduce elevated ketone body concentrations to approximately normal levels while tending to produce a lability in the elevated total body oxygen utilization from day to day that cannot be correlated always with minor fluctuations in the chemical analyses of blood or urine.

The possibility that one of the manifestations of the fatty liver secondary to deficiency of pancreatic lipotropic substance might be an elevated rate of oxygen consumption was suggested by the recent publication of Kessler, Liebler, Bronfin,

and Sass (28) who demonstrated elevated splanchnic and total oxygen consumptions resulting from fatty infiltration of the liver in alcoholic patients. The two lipotropic agents used in this investigation were administered to dogs in the dosage found effective in preventing fatty infiltration of the liver by Prohaska, Dragstedt, and Harms (29). Unfortunately no distinct relationship was found to exist between oxygen consumption and the fat content of the liver since values of the former frequently were elevated without appreciable liver fat being present. However, massive infiltration of the liver with fat invariably was associated with considerable elevation in rate of oxygen consumption. These findings are in general agreement with those of Bogoch, Casselman, Kaplan, and Bockus (30) who after meticulous clinical study of hepatic function in diabetes mellitus conclude that "no correlation could be established between the degree of fatty metamorphosis of the liver and any other histochemical or laboratory observation."

CONCLUSIONS

Total body oxygen consumption persistently increased from 20 to 50 per cent in the presence of the diabetes mellitus induced in dogs by phlorhizin, alloxan or pancreatectomy; the magnitude of the increase was associated with the severity of the diabetes. Insulin control of alloxan diabetes returned oxygen consumption to the normal range. Insulin control of the diabetes following pancreatectomy regularly decreased total oxygen consumption but did not, except on occasion, lower it to the prediabetic level regardless of whether choline or beef pancreas was supplied.

Hypophysectomized pancreatectomized dogs manifest a mild but labile diabetes mellitus with little tendency for development of ketosis or fat accumulation in the liver. Minimal rates of oxygen utilization in a series of Houssay animals were obtained while the diabetes had undergone a spontaneous remission and did not exceed rates for normal dogs under basal conditions. Periods of diabetic exacerbation were characterized by elevated rates of oxygen consumption.

The mean absolute increase in splanchnic oxygen uptake in a series of pancreatectomized dogs was found to equal or exceed simultaneously measured increases in the total oxygen consumption and thus roughly account for the elevated rate in total metabolism. Insulin in combination with choline was found slightly more effective than insulin with beef pancreas or insulin alone in its ability to restore elevated rates of oxygen consumption to normal in pancreatectomized dogs. No direct correlation was drawn between the total lipid content of the liver and the splanchnic oxygen consumption except that massive fatty infiltration of the liver was accompanied by maximal elevations in splanchnic and total oxygen utilization.

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HEAT STABILITY OF PROTEIN SOLUTIONS OBTAINED FROM HUMAN PLASMA BY DIFFERENT ETHANOL CONCENTRATIONS AND TEMPERATURES[1]

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In a previous publication we reported a method for the preparation of a heat stable human plasma protein solution (1). The method was a modification of that of Cohn, Strong, Hughes, Mulford, Ashworth, Melin, and Taylor (2) and used ethanol-water mixtures at low temperatures for the separation of the heat labile crude fibrinogen fraction (Fraction I) and gamma globulin fraction (Fraction II + III) from normal human plasma. The supernatant fluid following the removal of the crude gamma globulin fraction was dried from the frozen state and reconstituted to plasma volume with distilled water. Heating of this material for 10 hours at 60° C. changed the optical density at 550 mμ and the electrophoretic distribution very little. Electrophoretically, the heated material was 80 per cent albumin, 16 per cent alpha globulin and 4 per cent beta globulin. Heating of the solution for 10 hours at 60° C. caused the formation of a small amount of a very fast moving component in the ultracentrifuge. The remainder representing approximately 90 per cent of the starting protein concentration was homogeneous and had an uncorrected sedimentation constant of approximately 4.0×10^{-13}.

The present communication concerns the heat stability of human plasma protein solutions remaining following the precipitation of the Fraction II + III proteins from normal human plasma by different ethanol concentrations and temperatures.

METHODS AND RESULTS

Plasma used in this study was furnished by the American Red Cross through its Regional Blood Centers at Omaha, Nebraska and Springfield, Missouri. The plasma was pooled at the Blood Center in two-liter bottles and ten liters were shipped to us each week. No attempt was made to keep the plasma cold while en route. At the laboratory the plasma was stored at — 18° C. until the fractionation procedure was started.

The procedure for separation of the Fraction I and Fraction II + III proteins from human plasma was exactly the same as that described previously (1). Only the ethanol concentration and temperature for separation of the Fraction II + III proteins were changed. At least 8 liters of plasma were used in each preparation by each method. Table I shows the ethanol concentrations and temperatures that were used to separate the Fraction II + III proteins from the supernatant fluid of Fraction I. In all methods except Method S as shown in Table I the crude fibrinogen fraction, Fraction I, was separated from plasma at 8 per cent ethanol, pH 7.2 ± 0.2, ionic strength 0.14 and temperature 0° C. ± 0.5° C. The supernatant fluid obtained after removal of Fraction II + III by each method was dried from the frozen state. The dry powder was the material used in this investigation.

In all methods except Method S the pH and ionic strength for the separation of Fraction II + III were near 6.8 ± 0.05 and 0.12, respectively. In Method S the pH and ionic strength were 6.9 ± 0.05 and 0.133, respectively.

The electrophoretic distribution of the plasma protein preparations

The electrophoretic distribution of the plasma protein preparations obtained by Methods A, B, C, F, and S is shown in Table II.

The protein distribution of the plasma protein mixture was determined using the Klett Electrophoresis apparatus. The dry powder obtained by each method was dissolved to protein concentrations of 1 to 2 per cent in veronal citrate buffer of 0.1 ionic strength and pH 8.6 and dialyzed against this buffer.

Table II shows that the plasma protein mixtures prepared by Methods A, B, and C were approximately the same electrophoretically. All contained approximately 80 per cent albumin, 13 to 14 per cent alpha globulin, 5.6 per cent beta globulin, no gamma globulin and no fibrinogen. Methods F and S preparations showed more alpha and beta

[1] This investigation was supported in part by research grant (H1229) from the National Heart Institute of the National Institutes of Health, Public Health Service, in part by a grant from the Department of the Army and in part by the University of Kansas.

TABLE I

Ethanol concentrations and temperatures used for the separation of the Fraction II+III proteins

Method	95 per cent ethanol added	Final ethanol concentration	Temperature
	*ml./liter**	*per cent*	*° C.*
A	234	25	-5 ± 0.5
B	234	25	0 ± 0.5
C	160	20	-5 ± 0.5
F	160	20	0 ± 0.5
S	†	11	0 ± 0.5

* Ml. per liter of the supernatant fluid following separation of Fraction I.

† 131 ml. of 95 per cent ethanol were added directly to each liter of plasma. Fraction I was not removed beforehand.

globulin and contained gamma globulin. Occasionally fibrinogen was present also. The increase in globulin and fibrinogen concentrations resulted in a relative decrease in the electrophoretic concentration of albumin, as albumin was soluble for the most part under the conditions used in all methods studied.

The effect of heating for 10 hours at 60° C. on the electrophoretic patterns of the plasma protein solutions

The supernatant fluid following the separation of Fraction II + III by each method was clarified by filtration through Hormann D-8 pads, dried from the frozen state and reconstituted to plasma volume with distilled water. Plasma protein solutions containing either added 0.008 M sodium acetyl-DL-tryptophanate (AT) or 0.008 M sodium caprylate (Cap) were prepared also. Both stabilized as well as non-stabilized solutions were prepared such that the pH was 6.8 ± 0.2.

Each solution was sterilized by filtration through Hormann D-3, D-8, and D-10 filter pads placed into sterile Coleman Jr. Spectrophotometer tubes and capped with sterile rubber stoppers. Some of the tubes were heated for 10 hours at 60° C. while others not heated were used as control tubes. Following heating a few tubes of the heated solution were dried from the frozen state, reconstituted to protein concentrations of 1 to 2 per cent in veronal citrate buffer of 0.1 ionic strength and pH 8.6, dialyzed, and examined in the electrophoresis apparatus. Optical density measurements were made on the remaining tubes. The effect of 10 hours heating at 60° C. on the electrophoretic patterns is shown in Figure 1. The top row (U) represents the unheated control solutions, the bottom row (H) the solutions heated for 10 hours at 60° C.

Figure 1 shows that heating the solutions for 10 hours at 60° C. resulted in the formation of a new electrophoretic component having a mobility close to that of α_2-globulin. The size of this component was dependent upon the concentration of ethanol and the temperature used in preparation of the plasma protein mixture. Method A solutions had the smallest amount of the new component (Figure 1 AH) while Method S solutions had the largest amount (Figure 1 SH).

Except for the large new component the electrophoretic distribution of the proteins in solutions A, B, C, and F following heating for 10 hours at 60° C. was approximately the same as that of the unheated samples shown in Table II. There were slight reductions in albumin and beta globulin. Those components affected most by heating were the alpha globulins. Electrophoretically the protein distribution in the Method A solutions following heating for 10 hours at 60° C. was albumin 81.0 per cent, alpha globulin 14.0 and beta globulin 4.2. In both the B and the C solutions

TABLE II

The electrophoretic distribution of the plasma protein preparations obtained by Methods A, B, C, F, and S

Method and number of preparations	Approximate protein yield per 100 ml. plasma	Average electrophoretic distribution			
		Albumin	α-globulin	β-globulin	γ-globulin
	grams	*per cent*	*per cent*	*per cent*	*per cent*
A 3	3.4	81.6	12.8	5.6	0
B 4	3.5	79.6	14.6	5.5	*
C 3	3.9	80.2	14.5	5.3	0
F 3	4.3	73.6	18.8	5.4	2.2
S 2	4.9	65.7	20.1	4.4	8.8†

* One preparation was 1.3 per cent in γ-globulin.

† One preparation was 2.8 per cent in fibrinogen.

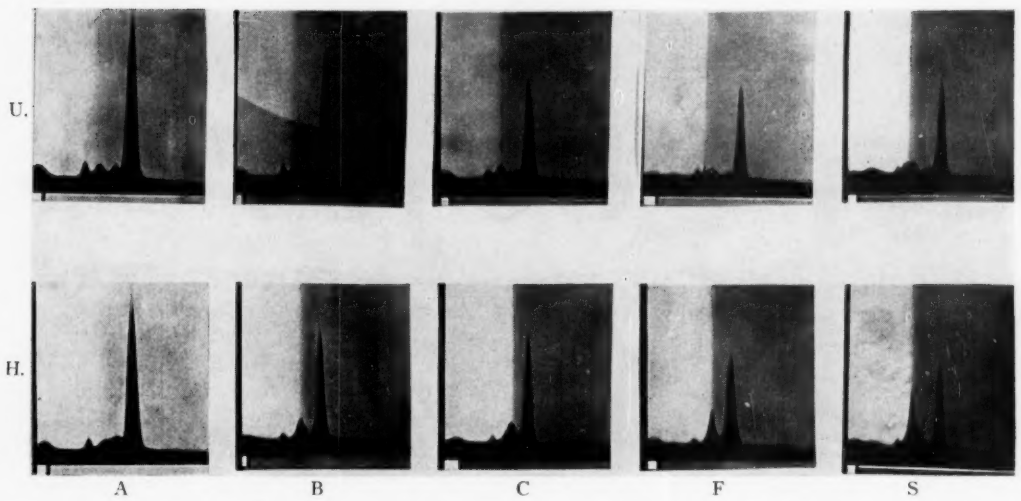

FIG. 1. THE ELECTROPHORETIC DIAGRAMS OF THE PLASMA PROTEIN SOLUTIONS

The upper row (U) represents the unheated solutions, the lower row (H) the solutions heated for 10 hours at 60° C. Each solution was made to 1 or 2 per cent in protein in veronal citrate buffer of 0.1 ionic strength and pH 8.6. The pictures above were taken between 120 to 150 minutes after starting the run.

albumin was 70 to 80 per cent, alpha globulin 14 to 24 and beta globulin 3.4. In the F solutions albumin was on the average 71.4 per cent, alpha globulin 23.9 and beta globulin 3.5.

The greatest effect on the electrophoretic distribution following heating for 10 hours at 60° C. was shown by the Method S solutions. The average protein distribution of the S material before heating (Table II) was albumin approximately 65.7 per cent, alpha globulin 20.1, beta globulin 4.4 and gamma globulin 8.4. One preparation was 2.8 per cent in fibrinogen. Following heating of the S solutions albumin was only 32.7 per cent in one solution and 53.3 per cent in the other. Alpha globulin was 40 per cent in one and 63.2 in the other. Beta globulin was approximately 2.2 per cent and gamma globulin 2.9 per cent in both solutions.

The addition of either 0.008 M sodium acetyl-DL-tryptophanate or 0.008 M sodium caprylate had little effect on the electrophoretic patterns of any of the solutions heated for 10 hours at 60° C. In many solutions there was an indication that acetyl-DL-tryptophanate did reduce the amount of the new component due to heating the solutions for 10 hours at 60° C. Both acetyl-DL-tryptophanate and caprylate have been demonstrated to be effective stabilizers of normal human albumin (3, 4).

The effect of heating for 10 hours at 60° C. on the optical density of the plasma protein solutions

Optical density readings of both the heated and control tubes were made at 550 mμ in the Coleman Jr. Spectrophotometer. The optical density

TABLE III

The effect of heating the plasma protein solutions for 10 hours at 60° C. at pH 6.8 on the optical density at 550 mμ

Method and number of preparations	Stabilizer	Average optical density at 550 mμ		Average optical density change due to heating
		Unheated	Heated 10 hrs. at 60° C.	
A 3	None	0.192	0.200	0.008
3	AT	0.200	0.206	0.006
3	Cap	0.186	0.184	−0.002
B 4	None	0.177	0.195	0.018
4	AT	0.168	0.185	0.017
4	Cap	0.169	0.177	0.008
C 3	None	0.241	0.259	0.018
3	AT	0.263	0.285	0.022
3	Cap	0.216	0.254	0.038
F 3	None	0.234	0.276	0.042
3	AT	0.249	0.293	0.044
3	Cap	0.204	0.280	0.076
S 2	None	0.365	0.473	0.108

values shown in Table III are average values for the number of preparations made by each method. For each preparation between 15 and 30 Coleman Jr. tubes of unheated solution and between 40 and 60 tubes of heated solution were read at 550 mμ in the Coleman Jr. Spectrophotometer. Table III shows the effect of heating the plasma protein solutions for 10 hours at 60° C. on the optical density at 550 mμ.

The optical density changes due to heating at 60° C. for 10 hours indicate that the plasma protein solutions of Methods A, B, and C were approximately the same. Table III shows that following heating at 60° C. for 10 hours solution A increased in optical density from 0.192 to 0.200, solution B from 0.177 to 0.195 and solution C from 0.241 to 0.259. Solutions prepared by Method F showed an optical density change due to 10 hours heating at 60° C. of 0.042. On gross examination following heating the F and S solutions were noticeably more hazy than the A, B, and C solutions.

The difference in initial optical density readings (Table III) may be explained partially by the protein concentrations (Table II). Since each solution was made to plasma volume, the A, B, C, F, and S solutions were approximately 3.4, 3.5, 3.9, 4.3, and 4.9 per cent in protein, respectively, as measured by the biuret method (5). Whether the change in optical density due to heating the solutions for 10 hours at 60° C. can be explained by difference in protein concentration has not been demonstrated conclusively. When 5, 6, and 7 per cent protein solutions were prepared from Method A material, the optical density changes following heating were only 0.01, 0.03, and 0.035 respectively. Method S solutions changed from an initial optical density of 0.365 to 0.473 or 0.108 on heating for 10 hours at 60° C.

The addition of either 0.008 M sodium acetyl-DL-tryptophanate (AT) or 0.008 M sodium caprylate (Cap) as stabilizers had little effect on the optical density change of solutions heated for 10 hours at 60° C (Table III). The optical density changes of solutions containing these substances were about the same as those containing no added stabilizer. Preliminary evidence (6) indicates that both tryptophanate and caprylate were effective in solutions heated for at least 75 hours at 60° C. Solutions containing no added stabilizer and heated at 60° for 75 hours or more showed a greater change in optical density than did the same solutions containing either added tryptophanate or caprylate.

The effect of heating for 10 hours at 60° C. on the ultracentrifugal sedimentation diagrams of the plasma protein solutions

Heated and unheated samples of plasma protein solutions prepared by Methods A, B, C, F, and S were studied in the Spinco Ultracentrifuge Model E. Each solution was diluted with 0.15 M NaCl such that the protein concentration was approximately 1.0 per cent. For each solution the heated and unheated samples were run at the same time using both cells of the rotor. Each solution was run at full speed of 59,700 RPM for 90 to 100 minutes and pictures were taken usually at 2, 5, 10, 20, 30, 40, 50, 60, 80, and 90 minutes after full speed of the rotor had been attained. As the rotor approached full speed pictures were taken also at 45,000 RPM and 57,000 RPM. Figure 2 shows the ultracentrifuge diagrams of the plasma protein solutions prepared by Methods A, B, C, F, and S. Column 1 represents pictures taken at the time the rotor had reached full speed, column 2 those taken 20 to 40 minutes after full speed and column 3 those taken 80 to 90 minutes after full speed. The upper diagram in each picture represents the solution heated for 10 hours at 60° C. and the lower diagram the unheated control solution.

A very fast moving heterogeneous component appeared in all solutions heated for 10 hours at 60° C. as the rotor neared full speed (arrow, upper diagrams, A1, B1, C1, F1, and S1). Very little was seen in the unheated solutions. The size of the component increased as the ethanol concentration and temperature used in preparation were decreased and increased, respectively (Method A to Method S).

Figure 2 shows that each of the unheated solutions had a small amount of fairly homogeneous material which appeared in the diagrams after the very fast moving component of the heated solutions had disappeared (arrow, lower diagrams, A2, B2, C2, F2, and S2). None of this component appeared in diagrams of heated solutions. It is suggested that during 10 hours' heating at

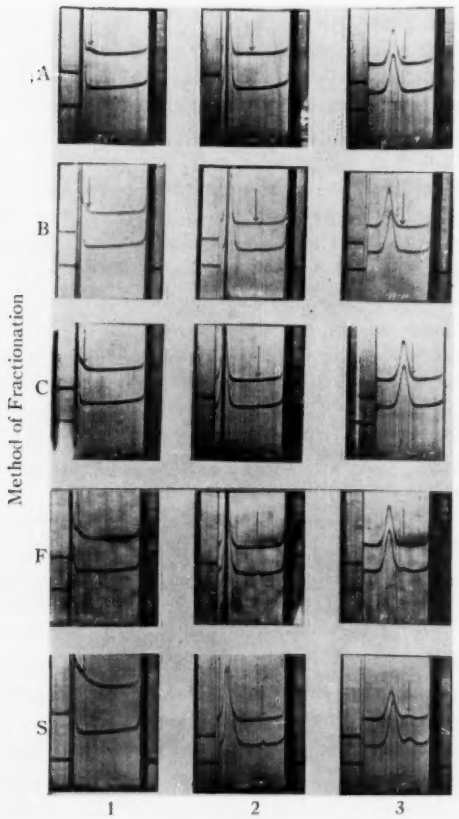

FIG. 2. THE ULTRACENTRIFUGAL SEDIMENTATION DIAGRAMS OF THE PLASMA PROTEIN SOLUTIONS

The upper diagram in each picture represents the heated solution, the lower the unheated solution. Pictures in column 1 were taken at the time the rotor reached full speed of 59,700 RPM, those in column 2, 20 to 30 minutes after full speed, and those in column 3, 80 to 90 minutes after full speed.

60° C. this component became part of the very fast moving material.

Later in the ultracentrifuge run another component appeared in both the heated and unheated samples of the F and S solutions (arrows, upper and lower diagrams, F3 and S3). This component was hardly discernible in the diagrams of the solutions prepared by Methods A, B, and C (arrows, A3, B3, and C3). In the heated samples of F and S solutions this component was smaller than it was in the unheated samples. This difference suggests that during 10 hours' heating at 60° C. a portion of this component became part of the very fast moving material.

The effect of 10 hours' heating at 60° C. on the main component of the plasma protein solution is shown in Figure 2. The greatest effect was in the S solutions (upper diagram, S3). The main component in the S solutions following 10 hours heating at 60° C. was markedly less than that of the unheated S solutions. The main components of the A, B, C, and F solutions were less following heating for 10 hours at 60° C. than they were in the unheated solutions. A rough calculation of the total areas of all components in both the heated and unheated solutions indicates that the size of the very fast heterogeneous component in heated solutions accounted for the decrease in area of each of the other components.

The extent to which the main component (Figure 2, A3, B3, C3, F3, and S3) was reduced when the solutions were heated for 10 hours at 60° C. is shown in Table IV. The area of the main peak of each of the heated and unheated samples was determined from the ultracentrifuge pictures taken at 60, 80, and 90 minutes after the rotor had reached full speed of 59,700 RPM. The three areas of the main peaks of the heated solutions were averaged as were the three areas of the unheated solutions. The ratio of the average area of the main component in the heated solution to

TABLE IV

The effect of heating the plasma protein solutions for 10 hours at 60° C. on the main component of the ultracentrifuge diagrams

Method	Stabilizer	Ratio of area of main component (heated) to area of main component (unheated)		
		Preparation 1	Preparation 2	Preparation 3
A	None	0.917	0.937	0.931
	AT	0.927	0.903	0.915
	Cap	0.911	0.972	0.915
B	None	0.778	0.909	0.901
	AT	0.943	1.000	1.005
	Cap	0.950	0.995	0.970
C	None	0.736	0.915	0.933
	AT	1.004	0.887	0.936
	Cap	0.881	0.964	0.751
F	None	0.678	0.965	0.907
	AT		0.938	0.893
	Cap	0.763	0.880	0.988
S	None	0.409	0.627	

that in the unheated solution for each of several preparations made by Methods A, B, C, F, and S, is shown in Table IV.

The addition of either 0.008 M sodium acetyl-DL-tryptophanate (AT) or 0.008 M sodium caprylate (Cap) had no effect on the ultracentrifuge diagrams of the solutions heated for 10 hours at 60° C. Table IV shows that the ratios of the areas of the main components of the heated versus the unheated solutions were essentially the same whether stabilizer was present or not.

DISCUSSION

Murray and Diefenbach (7) studied the effect of heating human plasma infected with the virus of serum hepatitis at 60° C. for various periods of time. They found that when plasma was heated at 60° C. for four hours or more there was an increase in turbidity and formation of a precipitate. van der Scheer, Wyckoff, and Clarke (8) found that when horse serum was warmed to about 65° C. it went through steps of opalescence and cloudiness with an increase in viscosity and plasticity. Electrophoretic examination showed that a new component was formed when the horse serum was heated for 15 minutes at 65° C. to 70° C. They designated this component as the C-component. Mobility was fairly constant in different preparations at approximately that of beta globulin. Evidence was presented that the C-component was formed at the expense of the albumin and the globulins. We have found that if all of the Fraction II + III proteins as well as the Fraction I proteins were not precipitated completely from plasma, the supernatant fluid following drying and reconstitution to plasma volume became cloudy and showed a new component in the electrophoresis apparatus following 10 hours' heating at 60° C. The electrophoretic component was quite similar to that seen by van der Scheer, Wyckoff, and Clarke (8). It had a mobility equal to that of α_2-globulin and was formed at the expense of both the albumins and globulins.

The amount of the component depended upon the conditions for precipitation of the Fraction II + III proteins. An ethanol concentration of 25 per cent at − 5° C. (Method A) resulted in maximum precipitation of the Fraction II + III proteins and least of the new component in the electrophoresis. The conditions in Method F (20 per cent ethanol and 0° C.) and Method S (11 per cent ethanol and 0°C.) gave less precipitation and a greater amount of the new electrophoretic component following 10 hours' heating at 60° C.

The plasma protein solutions prepared by Method A showed less optical density change and less of the very fast moving component in the ultracentrifuge following 10 hours' heating at 60° C. The solutions prepared by Methods F and S showed greater optical density changes and much more of the very fast moving component in the ultracentrifuge following heating. Both the F and S solutions were hazy after heating while the A, B, and C solutions were clear. The latter three remained clear after at least 50 hours' heating at 60° C.

Studies concerning the effect of storage at various temperatures on the optical density of the plasma protein solutions following heating for 10 hours at 60° C. are in progress. The optical density of both the stabilized and non-stabilized solutions changed very little after several months at 4° C., room temperature, and 37° C. Solutions prepared by Methods A, B, and C showed very little change in optical density after two to three weeks at 50° C., while those prepared by Method F increased 0.04 after one week at 50° C. Method S solutions increased approximately 0.04 in optical density in 24 hours at 50° C.

SUMMARY

1. Different ethanol concentrations and temperatures were used to remove the Fraction II + III proteins from human plasma in preparation of human plasma protein solutions.

2. When the ethanol concentrations were less than 25 per cent and the temperatures were higher than − 5° C., the plasma protein solutions following 10 hours' heating at 60° C. showed a new component in the electrophoresis patterns, an increased amount of a very fast moving component in the ultracentrifuge and a change in optical density.

3. When an ethanol concentration of 25 per cent and a temperature of − 5° C. was used, the resulting plasma protein solution following 10 hours' heating at 60° C. showed none of the new

component in the electrophoresis patterns, much less of the very fast moving component in the ultracentrifuge and much less change in optical density.

4. The stabilizers, acetyl-DL-tryptophanate and caprylate, had little stabilizing effect on the solutions heated at 60° C. for 10 hours.

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MYOCARDIAL TRANSFER OF SODIUM AND POTASSIUM: EFFECT OF ACETYL STROPHANTHIDIN IN NORMAL DOGS [1, 2]

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Although the hemodynamic events attending low-output congestive heart failure have been well defined, the biochemical changes in the myocardium responsible for these events and the response to cardiac glycosides remain largely unknown. Evidence to date suggests that in the failing heart there is normal substrate oxidation but an impairment of energy utilization (1). Recent data would indicate an altered state of the myocardial protein-complex, actomyosin (2), but whether this is the primary event in the evolution of congestive failure remains to be demonstrated.

Post-mortem tissue analyses for cation content of human heart muscle in failure have shown a lowered content of potassium (3, 4). Moreover, after digitalis, in therapeutic (5) and toxic doses (5, 6), the potassium content diminishes, although some disagreement over the former exists. That these changes may have functional significance is suggested by the work of Hajdu (7), who, from data obtained with a frog heart-muscle preparation, has inferred that the positive inotropism of digitalis is effected by the reduction in internal ionic content produced by the transfer of potassium from the cell.

The pronounced effect of digitalis on the mechanical efficiency of the failing myocardium (8, 9) suggests that this drug might provide data relating the movement of myocardial cations to cardiac hemodynamic function *in vivo*. Consequently, a study of these parameters was undertaken in the experimental animal, employing the rapid-acting digitalis analogue, acetyl strophanthidin.

METHODS

The experimental design of this study involves assumptions which appear sufficiently valid to justify their use. The coronary arterio-venous (A-V) differences of sodium and potassium were employed as a measure of their net uptake by, or release from, the myocardium. It is assumed that the shifting ions move quickly enough across the capillary to result in almost instant equilibration between interstitial fluid and effluent plasma (10). Whereas increased blood flow will dilute down or diminish A-V difference, decreased flow will augment it. Although no measurements of coronary blood flow have been made after strophanthidin, a separate study has revealed no change in myocardial oxygen A-V difference after this drug, suggesting that substantial changes in coronary flow do not occur. In other unpublished work, we have increased coronary blood flow by other experimental means and noted no change in electrolyte A-V difference. Thus we feel that the coronary flow changes accompanying strophanthidin dosage are insufficient in magnitude to bring about the electrolyte A-V differences to be reported.

Eight mongrel male dogs, weighing 14 to 21 kilograms, were anesthetized with sodium pentobarbital, 25 mg. per kg., supplemented as needed to maintain light anesthesia. A Cournand needle was placed in the femoral artery and single lumen catheters, filled with heparin, were placed via the external jugular vein in the coronary sinus to obtain blood samples and in the right auricle for injection of the drug. Heparin, 5 mg. per kg., was administered to prevent clotting, and pure oxygen delivered into an endotracheal tube to exclude the effects of hypoxia on plasma electrolytes (11).

Acetyl strophanthidin,[5] dissolved in 60 per cent ethyl alcohol (0.5 mg. per cc.) was infused into the right auricle in dosage of 0.05 to 0.1 mg. per kg. in volumes of

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[5] Kindly supplied by Dr. Kenneth G. Kohlstaedt, Medical Department, Eli Lilly and Company, Indianapolis, Indiana.

TABLE I

Electrolyte, hemodynamic and EKG findings

Dog No.	Dosage mg./kg.	Potassium mEq./L. Time	Fem-oral artery	Coro-nary sinus	Arterio-venous difference	Sodium mEq./L. Time	Fem-oral artery	Coro-nary sinus	Arterio-venous difference	Cardiac output cc./min./kg.	Pulse	Mean B.P.	Left vent. stroke work gm./M.	EKG
104	0.1	Control	3.69	3.62	+0.07	Control	156	155	+1.0		172	100		Normal
		Maximum change 7 min.	5.15	6.75	−1.60	Maximum change 3 min.	157	150	+7.0		174	180		Ventricular tachy-cardia
		Recovery 30 min.	4.00	3.90	+0.10	Recovery 30 min.	151	149	+2.0		180	140		Normal
106	0.05	Control	3.94	3.76	+0.18	Control	144	145	−1.0	162	162	130	32.0	Normal
		Maximum change 6 min.	4.81	5.30	−0.49	Maximum change 6 min.	156	148	+8.0	93	152	132	18.3	P wave diminished
		Recovery 27 min.	4.52	4.79	−0.27	Recovery 27 min.	151	149	+2.0		160	80		Normal
108	0.05	Control	4.68	4.66	+0.02	Control	147	154	−7.0	82	115	89	15.3	Normal
		Maximum change 15 min.	4.19	4.63	−0.44	Maximum change 7 min.	151	146	+5.0	113	117	83	19.5	P wave diminished and slight bradycardia
		Recovery 30 min.	4.04	3.88	+0.16	Recovery 17 min.	145	145	0.0		123	79		Normal
109	0.05	Control	4.80	4.67	+0.13	Control	159	164	−5.0	130	132	134	38.8	Normal
		Maximum change 6 min.	5.57	6.06	−0.49	Maximum change 18 min.	162	158	+4.0	97	96	120	33.0	P wave diminished
		Recovery 40 min.	4.37	4.12	+0.25	Recovery 40 min.	150	157	−7.0		101	111		Normal
113	0.05	Control	4.00	3.92	+0.08	Control	159	162	−3.0	132	186	124	18.7	Normal
		Maximum change 1 min.	4.49	5.46	−0.97	Maximum change 2 min.	154	146	+8.0	78	170	122	11.9	Nodal rhythm
		Recovery 6 min.	5.12	4.96	+0.16	Recovery 6 min.	153	153	0.0		205	110		Normal

TABLE I—*Continued*

Dog No.	Dosage *mg./kg.*	Potassium *mEq./L.* Time	Fem-oral artery	Coro-nary sinus	Arterio-venous difference	Sodium *mEq./L.* Time	Fem-oral artery	Coro-nary sinus	Arterio-venous difference	Cardiac output *cc./min./kg.*	Pulse	Mean B.P.	Left vent. stroke work *gm./M.*	EKG
114	0.05	Control	3.31	2.92	+0.39	Control	146	152	−6.0	191	136	102	33.6	Normal
		Maximum change 6 min.	4.18	4.76	−0.58	Maximum change 6 min.	147	137	+10.0	115	174	118	18.3	Nodal and ventricular rhythm
		Recovery 20 min.	3.77	3.78	−0.01	Recovery 20 min.	149	151	−3.0		120	121		Normal
115	0.05	Control	4.63	4.70	−0.07	Control	152	162	−10.0	113	198	162	17.2	Normal
		Maximum change 1 min.	5.28	6.68	−1.40	Maximum change 6 min.	156	159	−3.0	58	173	138	8.6	Nodal and ventricular rhythm
		Recovery 25 min.	5.26	5.28	−0.02	Recovery 25 min.	149	151	−2.0		148	88		Normal
116	0.05	Control	4.16	4.18	−0.02	Control	150	148	+4.0	109	160	98	11.7	Normal
		Maximum change 5 min.	5.27	6.18	−0.91	Maximum change 8 min.	146	140	+6.0	59	102	107	10.9	P wave diminished
		Recovery 15 min.	4.86	4.71	+0.15	Recovery 15 min.	143	142	+1.0		125	140		Normal
Averages		Control	4.15	4.05	+0.10	Control	151	154	−3.0	131	154	119	14.8	
Standard deviations					(±0.14)				(±4.5)					
Averages		Maximum change 6 min.	4.87	5.73	−0.86	Maximum change 6 min.	153	147	+6.0	88	133	117	11.2	
Standard deviations					(±0.44)				(±4.0)					

0.6 to 1.0 cc. over a period of 15 seconds. This rapid acting aglycone was selected so that its abrupt and short duration of action (12) might facilitate detection of electrolyte changes in coronary sinus blood, which might otherwise not be found with slower acting preparations. The hemodynamic properties of this drug appear to be qualitatively the same as the glycoside, Ouabain (13, 14), and it is presumed that its physical chemical properties are not essentially different.

Determination of cardiac output by a modification (15) of the Stewart-Hamilton dye method was made 10 to 20 minutes prior to drug injection and 10 minutes after, at which time, the hemodynamic effects of the drug are near maximal (12). Phasic and mean arterial pressures were obtained at 5-minute intervals with a Sanborn electromanometer and recorded on the Sanborn polyviso. Stroke work against pressure was calculated as the product of the mean femoral artery pressure and stroke output, and expressed in gram-meters (16). The electrocardiogram (Lead AV_F) was recorded on the polyviso just prior to giving the drug and at one-minute intervals for 15 minutes; thereafter, at 5-minute intervals until the control state was re-established. The respiratory rate was noted simultaneously.

Eight milliliters of whole blood were drawn in chemically clean, dry syringes simultaneously from the coronary sinus and femoral artery for control electrolyte samples, 2 to 10 minutes before drug administration. In four dogs, two sets of controls were drawn 10 to 15 minutes apart and were not significantly different. After strophanthidin injection through the right auricular catheter, 8 milliliter samples were taken from artery and coronary sinus at 2, 5, 8, 15, 30, 45, 60, and occasionally at 90, and 120 minutes. Timing was not precise in some instances, but remained simultaneous. After centrifuging at 1,500 r.p.m. for 20 minutes, plasma determinations of sodium and potassium were done on a Beckman flame photometer, with an error of less than 2 per cent on duplicate readings. The occasional hemolyzed samples were discarded. In four experiments, simultaneous blood samples from the artery and coronary sinus were secured in oiled syringes containing small amounts of heparin for immediate delivery to a Beckman glass-electrode pH meter maintained at constant temperature of 38° in the water bath, and in which duplicate readings agreed to 0.02 pH unit. Samples were taken during control periods and at 2, 8, and 15 minutes. Hematocrits were run on the same samples.

In determining the statistical significance of the electrolyte changes, utilizing standard methods (17), the maximum change in A-V difference of sodium and potassium was selected and compared with the control A-V difference. Intervening A-V differences generally represented increasing and decreasing gradations of these maximum values, as shown in Figure 1. The variability in time of occurrence of this maximum change is probably consistent with the variability of the hemodynamic time-response to cardiac glycosides. The figures for recovery, given in Table I, signify the first A-V difference values after the maximum response to approximate the control values.

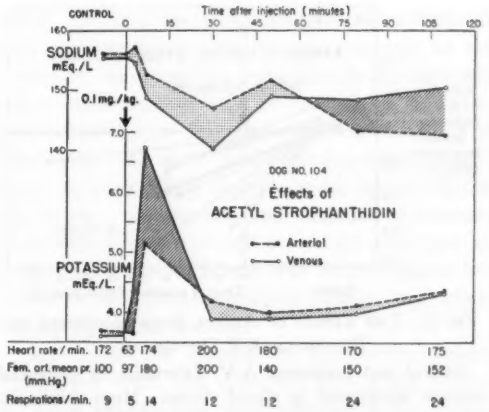

FIG. 1. MYOCARDIAL CATION EXCHANGE IN AN INDIVIDUAL EXPERIMENT TYPICAL FOR GROUP ILLUSTRATING SEQUENTIAL CHANGES AFTER ACETYL STROPHANTHIDIN

Venous refers to coronary sinus blood. Arterial refers to systemic artery. Stippling indicates positive A-V difference or myocardial uptake and diagonal shading indicates negative A-V difference or release of cation.

RESULTS

A typical individual experiment of this study is depicted in Figure 1, demonstrating the acuteness of the cation responses and their divergent movement as illustrated by the simultaneous positive sodium and negative potassium A-V differences. The reversal of these initial changes after 30 minutes, as seen in this experiment, was not a consistent finding in the group as a whole. The mean control potassium values for the group were 4.15 ± .52 mEq. per L. in the artery, and 4.05 ± .62 mEq. per L. in the coronary sinus, so that the mean A-V difference of 0.10 ± .14 was not significant in view of the error of determination. After the infusion of acetyl strophanthidin, there was, as shown in Figure 2, an abrupt loss of potassium in seven animals, a somewhat slower loss in the eighth, with the maximum change occurring in an average of six minutes for the group. The mean arterial potassium at this time was 4.87 ± 0.53 mEq. per L., and the coronary sinus value 5.73 ± 0.82 mEq. per L., so that a mean negative A-V difference of 0.86 ± .44 mEq. per L. was present, which, when subject with the control values to the t test, was found significant ($P = <$

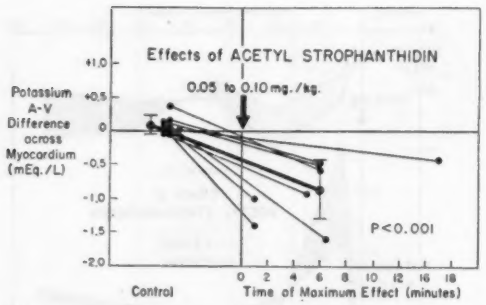

FIG. 2. THE EFFECT OF ACETYL STROPHANTHIDIN ON POTASSIUM A-V DIFFERENCE

Control and maximum A-V differences in individual animals represented by closed circles. Mean values for group represented by targets. Brackets indicate standard deviation.

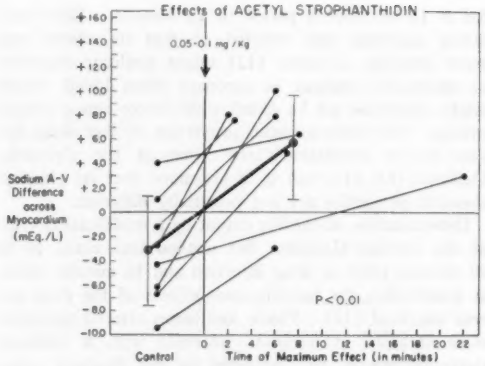

FIG. 3. THE EFFECT OF ACETYL STROPHANTHIDIN ON SODIUM A-V DIFFERENCE

Symbols same as in Figure 2.

.001). There was in general a less rapid reversion to control values of the potassium A-V difference (Figure 4), but variation was evident with a range of 6 to 50 minutes (Table I). The rise in arterial potassium was greater than could be accounted for by myocardial loss alone and presumably was derived from skeletal muscle in accord with previous tissue studies (3).

The mean value for the control arterial sodium was 151 ± 6 mEq. per L., and for the coronary sinus 154 ± 8 mEq. per L., so that a slight negative A-V difference of -3 ± 4 mEq. per L. was present. As seen in Figure 3, approximately concurrent with the potassium loss from the myocardium and despite the varied control values, there occurred increasing positivity of the A-V difference in each case, suggesting uptake of sodium. The mean A-V difference at the point of maximal change was $+6 \pm 4$ mEq. per L., which represents an increase of about 9 mEq. per L. over the control state. The arterial value was 153 ± 5 mEq. per L., the coronary sinus, 147 ± 8 mEq. per L. A comparison with the control figures gave a P value of < 0.01. Reversion to negligible A-V difference required, as with potassium, a variable time ranging from 6 to 50 minutes. Although more precise electrolyte measurement would be preferable, it would appear from the graphing of the composite values seen in Figure 4 that there was excess sodium ingress over potassium loss, suggesting that the ionic content of the myocardium increased, or that other cation was lost if this represents a complete exchange phenomenon.

The transient respiratory depression induced by the drug reduced ventilation by about 80 per cent for thirty seconds with rapid recovery and was accompanied by a decline in mean arterial pH from 7.46 to 7.36 in the 4 animals in which this was measured. In two dogs in whom there was no significant change in pH, electrolyte changes were of the same order as those with a substantial fall. Moreover, in another study (18), the same ionic changes were seen in animals in which respiratory alteration was prevented by vagotomy.

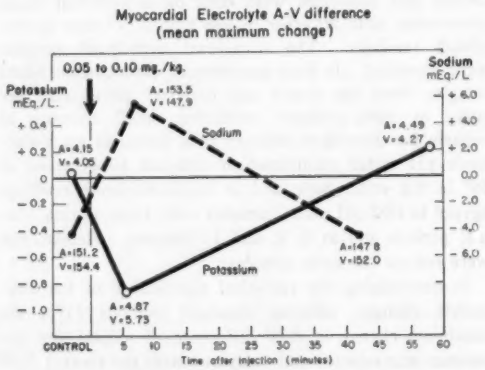

FIG. 4. COMPOSITE OF CONTROL, MAXIMUM AND RECOVERY MEAN A-V DIFFERENCE VALUES OF CATIONS FOR GROUP

Interrupted line, sodium; solid line, potassium. A refers to arterial and V refers to coronary sinus. Mean concentration of electrolytes in mEq. per L. at times indicated.

The difference between the coronary sinus pH and that of the arterial blood remained essentially the same during the experiment, except in the group of dogs with the more severe EKG changes, where the mean control pH A-V difference was + .05 and the maximum change was + .11 pH units, suggesting that the myocardium may release H + during the toxic response to strophanthidin.

The mean cardiac output fell from a control of 131 cc. per min. per kg. to 88 cc. per min. per kg. (Figure 5); the rise in one animal was apparently associated with an unsteady state, as the systemic arterial pressure was fluctuating throughout the experiment. This decrease was due to a diminished stroke volume of 14.8 cc. to 11.2 cc., and a decline in pulse rate from 154 to 133 beats per minute. Since the mean arterial blood pressure for the group remained the same, the stroke work change paralleled that of the stroke volume, decreasing from 23.8 gm. meters in the control to 17.7 gm. meters after acetyl strophanthidin.

Electrocardiographic changes in this group of animals appeared related to the magnitude of potassium transfer. Four dogs with minimal changes of bradycardia with or without P wave alteration had negative potassium A-V differences ranging from − 0.44 to − 0.91 mEq. per L. (average − .58). As the coronary sinus blood represents predominantly drainage from the left ventricle (19) and these animals gave no evidence of any electrocardiographic change referable to the ventricle, the potassium increment in the coronary sinus blood may be said to be derived from tissue exhibiting no electrical sign of toxicity. The four dogs with toxic electrocardiographic alterations manifested by ectopic beats, nodal and/or ventricular tachycardia had negative potassium A-V differences of − 0.58 to − 1.60 mEq. per L. (average − 1.14). There was no correlation between the electrocardiographic changes and myocardial uptake of sodium.

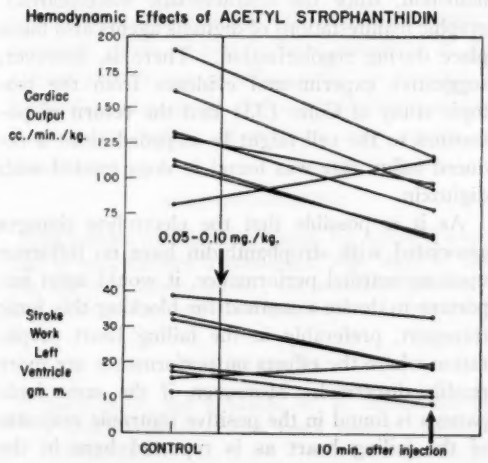

FIG. 5. THE EFFECTS OF ACETYL STROPHANTHIDIN ON CARDIAC OUTPUT AND STROKE WORK

No significant change in hematocrit values across the heart was observed during the course of these experiments, suggesting that there was no substantial transfer of water.

Digitalis preparations and ethyl alcohol may destroy the red cell membrane and release hemoglobin and potassium into the plasma (20). To exclude the alcohol vehicle as a cause of the observed electrolyte changes, 1 cc. of 60 per cent ethyl alcohol was injected into 3 dogs and produced no significant change in myocardial potassium A-V difference. Plasma hemoglobin determinations (21) were performed on the same plasma used for electrolyte analysis. Although there was a slight increase in plasma hemoglobin, the calculated increase in plasma potassium of .05 mEq. per L. as a result of the hemolysis would not significantly modify the plasma electrolyte concentration, as would be expected from the relatively low potassium content of 10 mEq. per L. in the canine red cell (19). Moreover, if there was any prozone effect on the red cells, it is unlikely that this would distort the arterio-venous difference values of potassium in such a manner as to contribute to the changes observed.

DISCUSSION

A direct action of cardiac glycosides on heart muscle has been demonstrated in a variety of ways, ranging from the improvement in contractile force of the fatigued papillary muscle of the cat (22), to the hemodynamic studies in the human subject with congestive failure in which increase in stroke output is the primary effect of the drug (8, 9). Since this improvement in the mechanical efficiency of the failing heart in low output failure occurs without significantly altering the supply

of chemical energy (23, 24), attention has been drawn to the direct action of digitalis upon the contractile protein.

That the contractile elements in heart muscle are the agents of this action has been suggested by Horváth, Király, and Szerb (25), who concluded that the basic action of the glycosides may be on the polymerization of actin prior to its union with myosin. In support of this general view, Robb and Mallov (26) have found that the actomyosin thread shortened more rapidly and to a greater degree under the influence of Ouabain. These reactions, however, might be secondary to changes in the ionic media since such changes have considerable influence upon the actomyosin system as has been suggested by Szent-Györgyi (27).

From the isolated heart muscle studies of Hajdu (7), it has been inferred that the positive inotropic effect of digitalis depends upon the diminished internal ionic content effected by the movement of potassium from the cell. Assuming that the only alterations occurring were in those ions measured, as suggested by tissue analyses (4), the normal dog heart in this study apparently underwent an increase in internal ionic content due to the greater net uptake of sodium over egress of potassium. It would appear possible that this alteration in the intracellular ionic atmosphere may represent the physico-chemical process responsible for the decline in stroke work in the normal animal (28, 29). That an increment in intracellular sodium associated with potassium loss may be deleterious to the heart is borne out by studies of Cannon, Frazier, and Hughes (30), in which this electrolyte situation was produced in the rat by dietary control, resulting in areas of focal necrosis mainly in the subendocardium.

While ionic changes may be but one of the determinants of myocardial contraction, and may exist to the extent observed here only under pharmacologic influence, it is quite possible that they participate in the physiologic control of cardiac output that attends altered ventricular filling pressure and cardiac muscle stretch. Hercus, McDowall, and Mendel (31) have found that stretch of the rat left ventricle promotes sodium loss and that increase in sodium extrusion may contribute to the efficiency of stretched muscle. The diminished efficiency found following impairment of the sodium extrusion mechanism would appear to parallel the findings in this study of strophanthidin in the normal animal, in which intracellular sodium accumulation was associated with diminished stroke work. While this ionic change in both instances may be but a by-product of the altered mechanical ability of the myocardium, the observation by Ranney (32) of a direct inhibitory effect of sodium at almost all concentrations in glycerol-extracted myocardial muscle would tend to support the view of an intimate relation between sodium accumulation and impaired mechanical ability.

The myocardial potassium loss seen in these animals, quantitatively greater in the four with toxic electrocardiographic changes, compares closely to that found by Conn (33) in normal dogs after digitoxin administration and amounted to 15 per cent of the total myocardial intracellular potassium by approximate calculation. Substantially the same myocardial potassium egress has occurred in patients reacting therapeutically to strophanthidin (34) so that the reduced potassium content of the untreated failing heart reported by other workers (3, 4) would appear to be enhanced by glycoside therapy.

The evidence provided by Wilde, O'Brien, and Bay (35) that there is potassium transfer across the heart with each cardiac cycle, egress of this ion occurring during repolarization, raises the question whether the potassium loss induced by glycosides is but an enhancement of this normal phenomenon, since the characteristic electrocardiographic manifestations of digitalis agents also takes place during repolarization. There is, however, suggestive experimental evidence from the isotopic study of Conn (33) that the return of potassium to the cell might be impeded since a reduced influx rate was found in dogs treated with digitoxin.

As it is possible that the electrolyte changes associated with strophanthidin have no influence upon myocardial performance, it would seem important to devise a method for blocking this ionic transport, preferably in the failing heart preparation where the effects on performance are more readily discerned. Moreover, if the same ionic pattern is found in the positive inotropic response of the failing heart as is reported here in the normal heart, then the lack of specificity of these ionic changes would militate against their having

a major role in the determination of myocardial performance.

SUMMARY

By simultaneous collection of blood samples from a systemic artery and coronary sinus, the myocardial transfer of sodium and potassium has been measured in eight anesthetized dogs before and after the administration of the digitalis analogue acetyl strophanthidin in doses of 0.05 to 0.1 mg. per kg. To relate cation transfer to cardiac performance, cardiac stroke work was assessed during the control period and at the time of maximal drug response.

There was no significant A-V difference of potassium during the control period. After strophanthidin, there was an abrupt increase in coronary sinus potassium with less rise in arterial potassium concentration, giving a mean maximum negative A-V difference of 0.86 ± 0.44 mEq. per L., indicating loss of potassium from the myocardium. Concurrently, there appeared to be a greater uptake of sodium with a positive A-V difference of 6.0 ± 4.0 mEq. per L. Both cation A-V differences resumed approximate control values at about 30 minutes. Animals with minor EKG changes after strophanthidin had less potassium loss than those with toxic EKG changes. No such correlation with sodium was found.

The stroke work of the left ventricle in this group of animals declined from a control value of 23.8 gm. meters to 17.7 gm. meters after strophanthidin. It is suggested that the negative inotropic effect of the digitalis analogues in the normal animal may be affected by the increase in internal ionic content of the myocardium due to the greater influx of sodium over egress of potassium.

ACKNOWLEDGMENT

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THE CAUSE OF HYPERCALCURIA IN SARCOID AND ITS TREATMENT WITH CORTISONE AND SODIUM PHYTATE [1, 2]

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Sarcoid is a chronic granulomatous disease of unknown etiology which may involve any or all organ systems (1). Harrell and Fisher (2) in 1939 first called attention to an elevated serum calcium in 6 of 11 patients with sarcoid. Other abnormalities noted were hyperproteinemia, due to hyperglobulinemia, and elevated serum alkaline phosphatase. The blood inorganic phosphorus was not decreased, a major point in the differential diagnosis with hyperparathyroidism. The urinary calcium excretion was not reported but we now know that hypercalcuria is common in sarcoid [3] and often the cause of renal stones which are usually composed of calcium oxalate.

The cause of hypercalcemia and hypercalcuria in sarcoid has been obscure. Schaumann (4) demonstrated sarcoid involvement of bone and indicated that this is found more commonly histologically than radiologically. Small "punched out" areas of bone destruction may be found in 15 to 29 per cent of patients (1), particularly in the phalanges but rarely in the long bones and skull as well. Even generalized bone demineralization has been reported (5) but most patients with sarcoid, including those patients with hypercalcuria of many years' duration, have minimal or no detectable bone disease. The common concurrence of skin involvement with bone involvement has been emphasized (1).

It has been suspected that the hyperglobulinemia in sarcoid might "bind" an excessive amount of calcium and thus account for hypercalcemia. If this extra moiety of calcium were bound and unionized it probably would not pass the glomerular filter and produce hypercalcuria. It is of interest that berylliosis, which may produce a clinical and pathological picture almost indistinguishable from sarcoid, also may be associated with hypercalcuria.[4]

Evidence is presented in this paper that the hypercalcuria in sarcoid is not due to bone destruction, but is due to a disturbed metabolic pattern closely resembling that of vitamin D overdosage.

METHODS

The data presented are derived from four complete balance studies; the analytical methods previously described (7) were used except that sodium and potassium were determined with a lithium internal standard flame photometer (8), magnesium by the Briggs' method (9), and urinary 17-ketosteroids by the micro method of Per Vestergaard (10). A single constant diet was used throughout each study rather than 3 alternating diets as previously described. Fasting blood samples were used for all studies.

CASE HISTORIES

In view of the highly variable clinical picture of sarcoid the case histories of our 3 patients are presented in some detail.

Case 1, A. M., M. G. H. No. 252484

This 37-year-old Lithuanian-born Jewish housewife was first seen in 1940 because of chronic cough of 2 years' duration. Positive findings on examination were cyanosis, mild generalized lymphadenopathy and splenomegaly. The lungs were extensively involved with nodular densities by x-ray. Tuberculin skin tests and guinea-pig inoculations of sputum were negative, as is the rule in

[1] These studies were supported in part by grants from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council, the United States Public Health Service, and the Rockefeller Foundation and were carried out in the Mallinckrodt Research Ward of the Massachusetts General Hospital.

[2] Presented in part before the American Society for Clinical Investigation, May, 1954.

[3] Dr. John Eager Howard (3) (Johns Hopkins Hospital) estimates that 20 to 30 per cent of patients with sarcoid present hypercalcemia.

[4] Dr. Harriet L. Hardy (6) (Massachusetts General Hospital) reports a 20 per cent incidence of kidney stones in berylliosis patients studied prior to 1952.

sarcoid. A lymph node biopsy was interpreted as consistent with sarcoid.

In 1941 the patient complained of right flank pain and a renal stone was demonstrated by x-ray. Hypercalcemia and hypercalcuria were demonstrated. For two months prior to the balance study (vide infra) the patient received three Haliver Oil capsules daily (total 300 units vitamin D).

The patient's serum calcium gradually returned to normal and remained normal after 1943 despite increasing hyperglobulinemia and pulmonary insufficiency. A renal stone was removed in 1948 and was found to contain primarily calcium oxalate with some calcium phosphate.

The patient died of cor pulmonale and pulmonary infarction in 1954.

Case 2, M. S., M.G.H. No. 793791

This 38-year-old Russian-born machinist first noted polyuria, polydipsia, pruritus, weight loss, and a scalp infection in March, 1952. Mild diabetes mellitus was diagnosed but diet and insulin therapy did not diminish his polydipsia. In June, 1952, he began to note photophobia, halos about lights and progressive blurring of vision. In November, 1952, he came to the Massachusetts Eye and Ear Infirmary where Dr. David Cogan found a curious granular superficial opacity just beneath the epithelium occupying practically the entire cornea of each eye but separated from both the upper and lower limbus by a 1 to 2 mm. clear zone. The uveal tract appeared normal. Because of the finding of hypercalcemia he was referred to us for study.

In addition to the eye findings there was fever, minimal brown pigmentation of the skin, and mild hepatosplenomegaly. The lungs were clear to physical examination and revealed by x-ray only a well healed calcified primary tuberculous complex in the right upper lung. The bones were normal. The tuberculin skin test (1:100) was negative. A liver aspiration biopsy revealed multiple granulomata with Langhans type giant cells but no evidence of caseation, findings consistent with a diagnosis of sarcoid. Biopsy of the corneal epithelium showed a positive von Kossa stain for calcium.

Since there was no known occupational exposure to beryllium, diagnoses of sarcoid and diabetes mellitus were made and the patient was transferred to the metabolic ward. For several months prior to study the patient had taken daily 1 capsule Vi-Syneral® and 2 capsules Lederplex®. The former is advertised to contain 1,000 units vitamin D.

Following 21 months' continuous therapy with oral cortisone and 6 months' therapy with sodium phytate, the patient's corneae almost entirely cleared and his vision sufficiently improved for him to return to his original occupation as a wire-extruder. Cortisone was discontinued in January, 1955; hypercalcemia returned abruptly in June, 1955. Combined therapy with cortisone and sodium phytate have diminished his hypercalcemia but failed to decrease his urinary calcium excretion below 400 mg. daily in the succeeding year.

Case 3, M. L., M.G.H. No. 445378

This 38-year-old Jewish fuel-oil salesman was born in Massachusetts and was well until 1944 when diffuse pulmonary fibrosis was discovered accidentally by x-ray examination. Tuberculosis was rendered unlikely by negative sputum examinations and negative tuberculin skin tests. Renal colic first occurred in 1946. The left kidney was removed because of nephrolithiasis and pyelonephritis in 1953. Granulomata consistent with sarcoid were found in a lymph node biopsy in 1951. Because of the recurrent nephrolithiasis (calcium oxalate) in the sole remaining kidney and B. Pyocyaneus pyelonephritis resistant to all antibiotics he was referred to us in December, 1953, by Dr. Melvin Lyons of Boston. The only history of vitamin D ingestion was of about 1,000 units daily (1 Theragran capsule) for 30 days six months prior to study.

Positive physical findings were brown pigmentation of the face, slight generalized lymphadenopathy and an enlarged right kidney. The lung findings by x-ray were consistent with extensive fibrosis. A single renal stone was noted by x-ray initially but the patient subsequently passed small stones weekly and even more frequently at times.

Following completion of the balance study the patient was maintained on daily sodium phytate therapy for 13 months, during which time his urinary calcium excretion was 30 to 90 mg. per 24 hours; he was stone-free and urine cultures were negative. Four months after discontinuing phytate therapy (in July, 1955) hypercalcemia returned; normal serum and urinary calcium excretion have again been maintained for the past 12 months with prednisone [5] therapy. In June, 1956, the patient developed a tumor of the right breast which was proved by excision to be benign gynecomastia.

RESULTS

Study 1. Patient A. M. (Figure 1)

This 36-day balance study is divided into 18 days on a very low calcium intake and 18 days on a medium calcium intake; the latter was achieved by the addition of 5 grams of calcium gluconate to the low calcium diet.

On the low calcium (estimated 60 mg.) constant intake the urinary calcium averaged 407 mg. and the fecal calcium averaged 23 mg., which gave a negative calcium balance of 370 mg. per day. On the moderate calcium intake of 560 mg. per day the urinary calcium averaged 456 mg., the fecal calcium 138 mg. and the negative calcium balance only 34 mg. per day. The patient was in nitrogen balance but in slightly negative phosphorus and calcium balance throughout the study. The serum

[5] Prednisone was generously supplied by Schering Corporation.

calcium was elevated and the serum phosphorus and serum alkaline phosphatase were normal. The fecal phosphorus was low throughout the study.

The observation that the calcium balance varied with the calcium intake was interpreted in 1941 (11) as favoring a metabolic cause for the hypercalcuria and hypercalcemia of sarcoid since varying the calcium intake would have had less effect on the calcium balance if bone destruction were the cause of the hypercalcuria. Unnoticed in 1941, but of greater significance, was the extremely low fecal calcium excretion. This suggests that one cause for the hypercalcuria may be increased absorption of calcium from, or decreased excretion into, the intestinal tract.

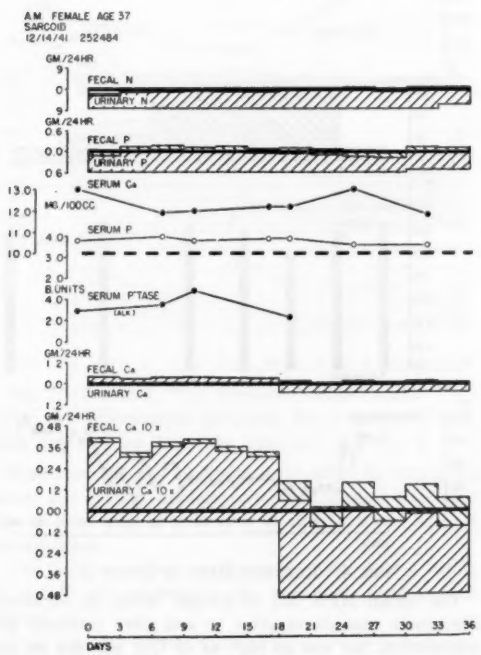


FIG. 1. STUDY 1, THE NITROGEN, PHOSPHORUS AND CALCIUM BALANCE DATA DURING LOW AND MODERATE CALCIUM INTAKES OF A. M.

In each balance figure the intake is measured down from the heavy zero baseline. The urinary and fecal excretion are charted up from the intake line. Negative balance is shown by a hatched area above the zero baseline and positive balance by a clear area below the zero baseline.

The relative scales for nitrogen, phosphorus, calcium and potassium represent the ratios of these substances in protoplasm and bone. The scales for sodium, chloride and magnesium were chosen arbitrariiy.

The dietary intakes in this study were calculated from dietary tables; diets in the other studies were analyzed. The fecal nitrogen was estimated to be 10 per cent of intake; fecal calcium and phosphorus were measured.

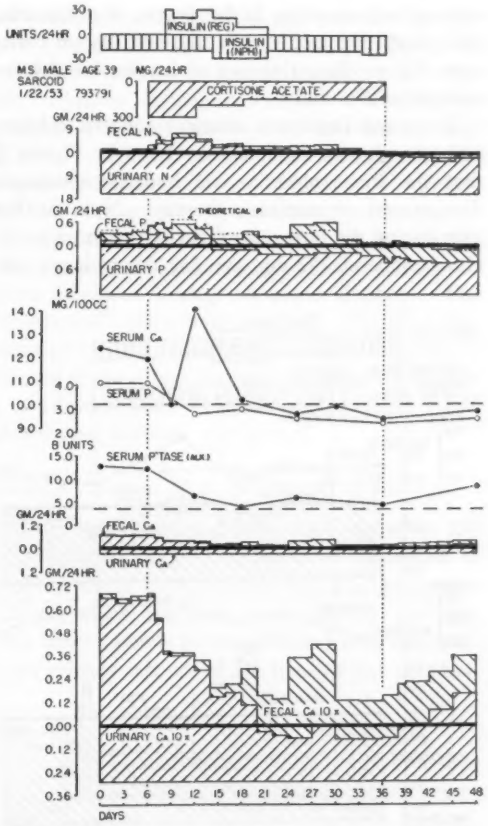


FIG. 2. STUDY 2, THE NITROGEN, PHOSPHORUS, AND CALCIUM BALANCE DATA DURING CORTISONE THERAPY OF M. S., A 39-YEAR-OLD MAN WITH SARCOID

The calcium data are recharted below for clarity on a 10 × larger scale.

The "theoretical " phosphorus balance is charted as a broken line on the "actual" phosphorus balance.

Study 2. Patient M. S. (Figures 2, 3, and 4)

This 48-day balance study consists of 6 days fore-control, 6 days on 300 mg. oral cortisone acetate [6] daily in divided doses, 6 days on 200 mg. cortisone, 18 days on 150 mg. cortisone and 12 days after-control off cortisone. NPH insulin, usually 20 units, was given daily and regular in-

[6] Cortisone acetate was generously supplied by Merck & Co.

sulin added according to the degree of glycosuria. An episode of hypoglycemia, the first day off cortisone, led to discontinuance of insulin for the remainder of the study.

The most important changes concern calcium and phosphorus metabolism. Note in Figure 2 that the hypercalcemia and hyperphosphatemia disappeared on cortisone therapy. Note further that during the fore-control period when on a calcium intake of 292 mg. per day the patient's uri-

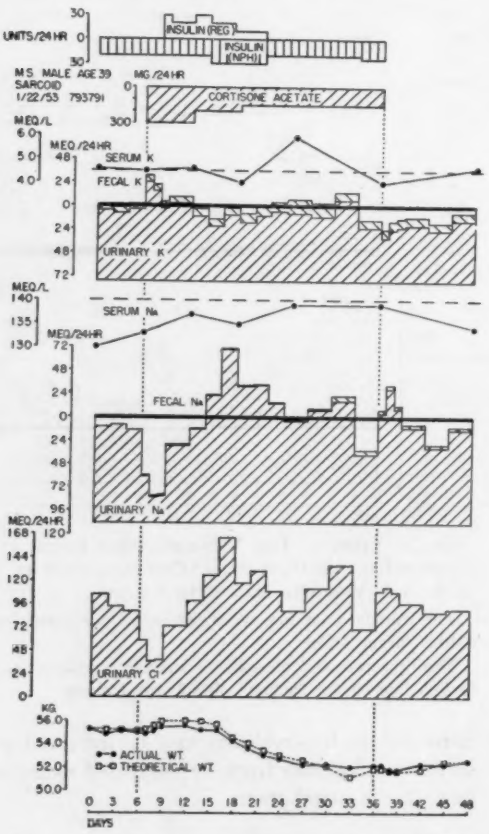


FIG. 3. THE POTASSIUM, SODIUM AND CHLORIDE BALANCE DATA, ACTUAL AND THEORETICAL WEIGHTS IN STUDY 2

Only urinary chloride is charted since fecal and dietary chloride were not measured.

The theoretical weight is that calculated from changes in nitrogen, potassium and sodium balances.

The urinary potassium increased abruptly the first two days on cortisone; the urinary potassium: nitrogen ratio exceeded the usual (2.7:1) protoplasmic ratio of these substances during these two days.

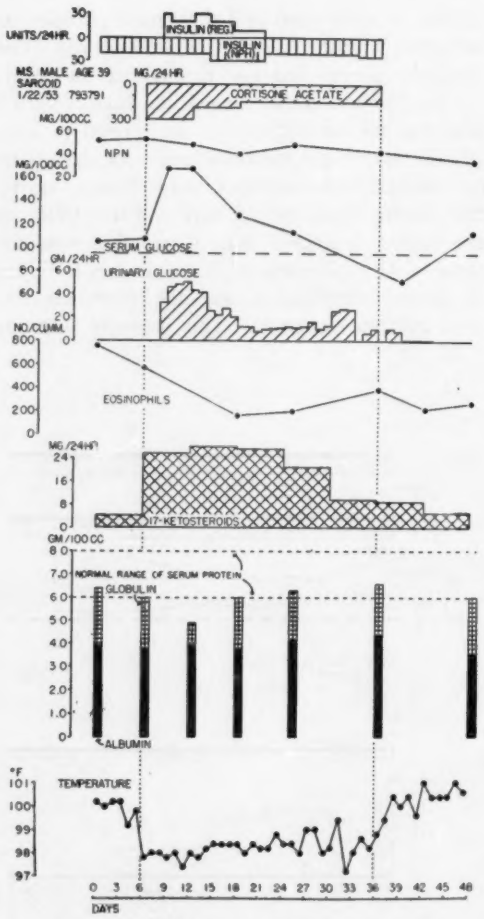


FIG. 4. FURTHER DATA OF STUDY 2

The serum NPN fell to normal levels on cortisone. Glycosuria was absent prior to and after cortisone administration, but was as high as 45 Gm. per day on the largest doses of cortisone. The rise in 17-ketosteroid excretion may represent excretion of cortisone metabolites and improvement in liver function (12). Evidence for the latter was the observed diminution in size of the liver and spleen and the fall in serum alkaline phosphatase to normal values during cortisone administration. The 4:00 P.M. daily oral temperatures fell to normal on the larger doses of cortisone, rose slightly when the cortisone dosage was reduced, and returned to fore-control levels on discontinuing cortisone; this finding suggested that the sarcoid process was still active at the end of the study. The serum proteins were normal, as determined by the Howe (13) method and showed no consistent changes[7] during cortisone therapy.

[7] Electrophoresis (courtesy of Dr. Karl Schmid) of the serum proteins gave the following results:

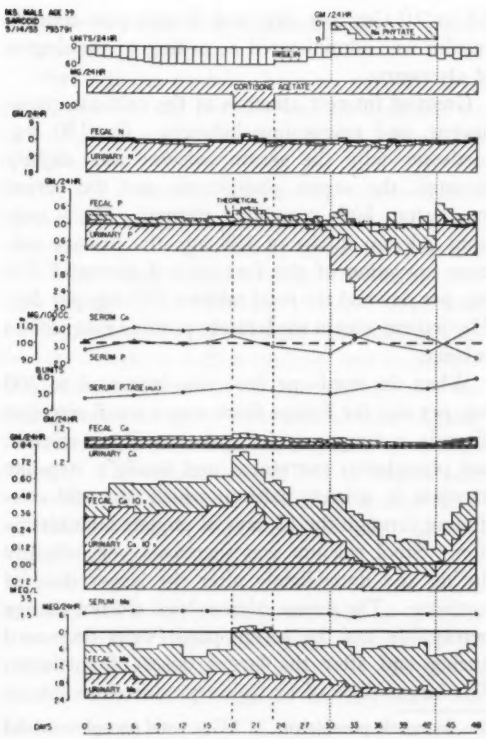


FIG. 5. STUDY 3, THE NITROGEN, PHOSPHORUS, CALCIUM AND MAGNESIUM BALANCE DATA DURING CORTISONE AND SODIUM PHYTATE TREATMENT OF M. S.

Note that 150 mg. cortisone daily failed to return the serum and urinary calcium to normal levels. Note also that on this dose of cortisone the fecal calcium was at normal levels.

Ten units crystalline insulin were given days 1 to 22, 43 and 45 plus protamine zinc insulin to total amount charted.

TABLE I

Results of serum protein electrophoresis on M.S.

	Albumin	Globulins					
		α_1	α_2	β_1	β_2	γ	
Normal values	55	5	9	13	7	11	%
M.S. pre-cortisone	52	6	18	7	6	11	%
M.S. post-cortisone	60	2	12	10	6	10	%

Dr. Milton B. Engel (University of Illinois) reported the patient's serum mucoproteins to be increased (41 mg. per cent carbohydrate residue, 5 mg. per cent tyrosine residue) before cortisone and little changed (38.3 mg. per cent carbohydrate residue, 4.9 mg. per cent tyrosine residue) after cortisone. He reports the normal value of the carbohydrate residue to be about 16 mg. per cent.

nary calcium averaged 930 mg. per day and the fecal calcium averaged only 23 mg. per day. The administration of cortisone produced a rapid steplike reduction in urinary calcium to a minimum value of 216 mg. per day, an increase in fecal calcium to 417 mg. per day, and a decrease in the negativity of the calcium balance. The urinary calcium rose again on discontinuing cortisone.

Note that the fecal phosphorus was low during the fore-control and increased when the fecal calcium increased during cortisone therapy. The "theoretical" phosphorus balance (7), *i.e.*, that explained by the nitrogen and calcium balances, is charted in the actual phosphorus balance in Figure 2 as a broken line. It agrees well with the actual phosphorus balance.

Study 3. Patient M. S. (Figures 5, 6, and 7)

M. S. was discharged from the hospital on 150 mg. cortisone daily. At home he continued to manifest slight hypercalcemia and marked hypercalcuria which averaged about 500 mg. per day. This latter value is to be contrasted with the minimum urinary calcium of 216 mg. per day noted on

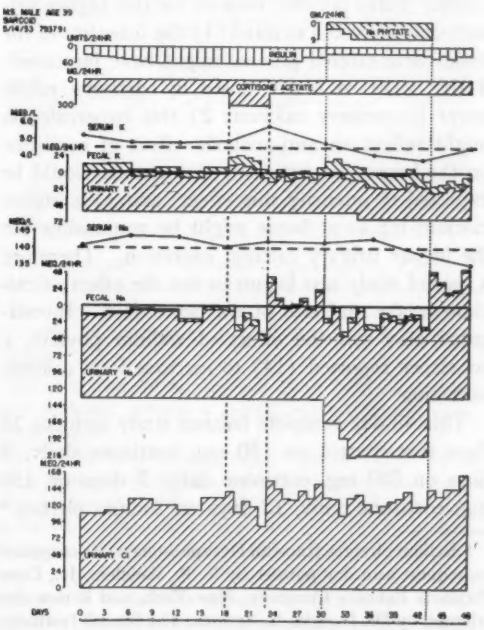


FIG. 6. POTASSIUM, SODIUM AND CHLORIDE BALANCE DATA DURING STUDY 3

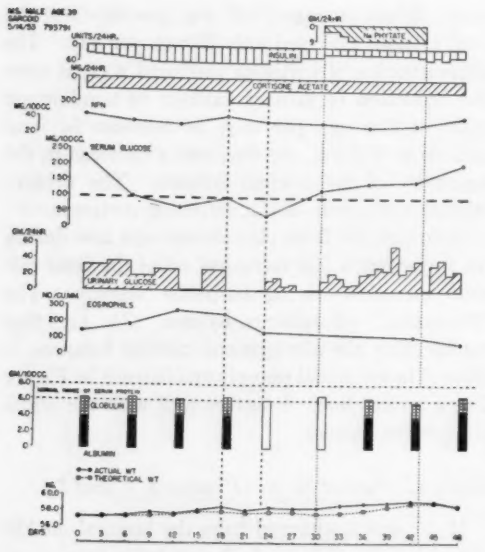


FIG. 7. FURTHER DATA OF STUDY 3

The open serum protein columns indicate that total protein only was determined.

the same dose of cortisone during the balance study. Three possible reasons for this higher urinary calcium come to mind: 1) the intensity of the underlying sarcoid process might have increased; if this were so larger doses of cortisone might lower his urinary calcium; 2) this hypercalcuria might reflect an anti-anabolic effect of cortisone on the bones; larger doses of cortisone would be expected to intensify this effect; and 3) a higher calcium intake at home might be responsible for the higher urinary calcium excretion. Therefore a second study was begun to test the effect of calcium intake and size of cortisone dose. Investigated also was the effect of sodium phytate, a substance reported (14) to increase fecal calcium excretion.

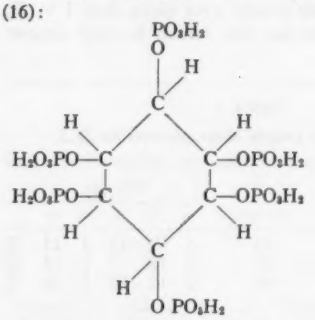
This 48-day complete balance study includes 18 days fore-control on 150 mg. cortisone daily, 5 days on 300 mg. cortisone daily, 7 days on 150 mg. cortisone daily, 13 days on sodium phytate [8] 3.6 to 9.0 Gm. per day, and 5 days post-control. Insulin was administered according to the degree of glycosuria.

Greatest interest attaches to the calcium, phosphorus, and magnesium balances. On 150 mg. cortisone daily the serum calcium was slightly elevated, the serum phosphorus and the serum magnesium both remaining normal. On a constant calcium intake of 178 mg. the urinary calcium excretion of the fore-control averaged 551 mg. per day and the fecal calcium 193 mg. per day. The patient was in moderately positive magnesium balance.

When the cortisone dose was increased to 300 mg. per day for 5 days there was a small nitrogen diuresis, a temporary increase in urinary calcium and phosphorus excretions, and finally a step-like decrease in urinary calcium which was still continuing through the 13 days of phytate administration. Stool calcium was increased very slightly during and immediately after the larger dose of cortisone. The serum calcium level did not change perceptibly but the serum phosphorus decreased during and after the larger doses of cortisone. The larger dose of cortisone produced an unsus-

[8] Sodium phytate (Inositol Hexaphosphate) was supplied on request as a dry powder by E. R. Weidlein, Jr., Corn Products Refining Company, New York, and is now distributed by Dr. Douglas B. Remsen, The Squibb Institute, New Brunswick, New Jersey. A 15 per cent solution in distilled water had a pH of 7.2. By assay each 100 Gm. sodium phytate powder contained 970 mEq. sodium, 3.88 Gm. inorganic phosphorus, 21.2 Gm. total phosphorus, and 4.8 Gm. unaccounted for which is probably water. By inorganic phosphorus is meant that amount reacting in the Fiske and Subbarow method (15) before ashing; by total phosphorus is meant that amount reacting in the Fiske and Subbarow method after wet ashing of the specimen. The difference (82 per cent) is assumed to represent phosphorus organically bound in the phytate molecule.

The theoretical formula for Phytic Acid is as follows (16):



$C_6H_{18}O_{24}P_6$

Mol. Wt. = 660

Assuming that the analyzed 3.88 Gm. inorganic phosphorus is a neutral mixture of $\frac{1}{4}Na_2HPO_4$ and NaH_2PO_4 contaminating the current sodium phytate preparation, the above analysis indicates approximately 8 mEq. Na per mM phytic acid at pH 7.2 and a molecular weight of sodium phytate of 836. The exact physical-chemical state of sodium phytate is not known.

tained increase in fecal magnesium but no change in urinary magnesium.

When sodium phytate, 3.6 Gm. per day for 2 days, then 9.0 Gm. per day was administered for 11 days there was a continued fall in urinary calcium to a low value of 120 mg. per day. That this further fall in urinary calcium was due to sodium phytate and not to the previous period on 300 mg. cortisone daily is supported by the observation that on discontinuing sodium phytate the urinary calcium rapidly rose to 502 mg. per day. The fecal calcium increased approximately 100 mg. per day the first 6 days on sodium phytate, was unchanged the remaining 7 days on sodium phytate, and was slightly less during the 5 days off sodium phytate. The calcium balance was less negative on sodium phytate. The serum calcium fell to normal and the serum phosphorus rose during sodium phytate; these changes were reversed when sodium phytate was discontinued.

On sodium phytate the total phosphorus intake increased 1,873 mg. per day; the fecal phosphorus increased 500 mg. and the urinary phosphorus increased approximately 1,000 mg. per day to account for a gain of 300 mg. per day in phosphorus balance.

The fecal magnesium increased approximately 4 mEq. and the urinary magnesium decreased approximately 5 mEq. on sodium phytate.

In regard to the three possible reasons why M. S.'s urinary calcium was higher at home on 150 mg. cortisone than it had been during the first study on the same dose of cortisone, one can conclude that this dose of cortisone had corrected the excessive calcium absorption but had only partially corrected the high urinary calcium excretion. The high urinary calcium was not due to the higher calcium intake at home since the urinary calcium did not decrease on a very low calcium intake in this second study. The hypercalcuria did not reflect an anti-anabolic effect of cortisone on the bones since increasing the cortisone dosage decreased rather than increased the urinary calcium. Thus it seems that either the severity of the sarcoid and its associated abnormality in calcium metabolism had increased or larger doses of cortisone were required to suppress sarcoid than to maintain this suppression. Indeed when cortisone therapy was reinstituted after the first study there was no preliminary period on larger doses.

In this study sodium phytate decreased the urinary calcium but did not increase the fecal calcium. The decrease in urinary calcium may have been due to increased phosphorus intake (see below). Sodium phytate did decrease urinary magnesium by approximately the same amount it increased fecal magnesium.

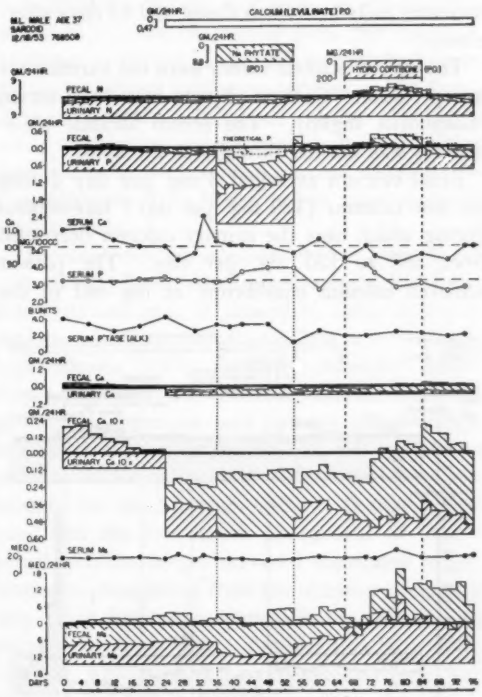


FIG. 8. STUDY 4, NITROGEN, PHOSPHORUS, CALCIUM AND MAGNESIUM BALANCE DATA DURING LOW AND MEDIUM CALCIUM INTAKES, SODIUM PHYTATE AND HYDROCORTISONE THERAPY OF M. L., A 37-YEAR-OLD MAN WITH SARCOID

The very high urinary calcium at the beginning of the study probably reflects a higher calcium intake prior to this study.

Patient M. L. was in nitrogen balance except for a period of negative nitrogen balance while on hydrocortisone. He was in slightly negative phosphorus balance during the low calcium intake fore-control, in slightly positive phosphorus balance on the higher calcium intake fore-control, in positive phosphorus balance on sodium phytate and in negative phosphorus balance on hydrocortisone. Fecal phosphorus was extremely low during the control periods and increased when the fecal calcium increased during sodium phytate and hydrocortisone administration.

Study 4. Patient M. L. (Figures 8, 9, 10 and Table III)

This 96-day complete balance study consists of 24 days on a low calcium intake (120 mg.), 12 days on a higher calcium intake (590 mg.) which was then continued for the rest of the study, 18 days on sodium phytate powder 8.8 Gm. per day, 12 days off medication, 18 days on 200 mg. hydrocortisone daily in divided doses, and 12 days after-control.

The serum calcium values were too variable for interpretation. Sodium phytate raised the serum phosphorus slightly. The serum alkaline phosphatase was normal throughout the study.

Fecal calcium averaged 8 mg. per day during the low calcium (120 mg. per day) fore-control during which time the urinary calcium decreased from 350 to 130 mg. per day. The patient achieved calcium equilibrium at the end of the low calcium fore-control and was in about 200 mg. positive calcium balance on the higher calcium intake, except during and immediately following hydrocortisone administration.

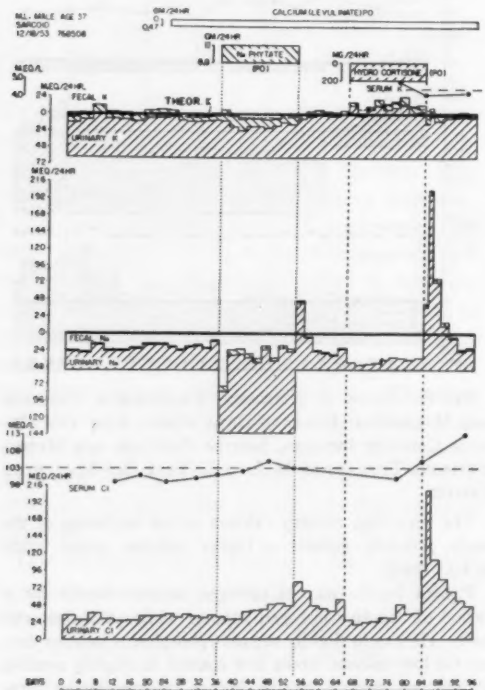


FIG. 9. POTASSIUM, SODIUM AND CHLORIDE BALANCE DATA DURING STUDY 4

Urinary potassium excretion increased abruptly the first two days on hydrocortisone; the urinary potassium: nitrogen ratio during these two days was greater than the normal protoplasmic ratio of these substances. (See Table III.)

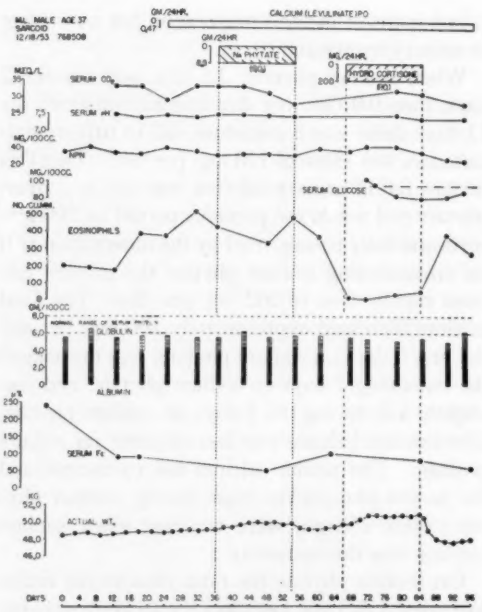


FIG. 10. OTHER DATA OF STUDY 4

The serum CO_2, pH, NPN and proteins were essentially constant throughout the study. The initial high serum iron value was not confirmed by other determinations during the fore-control.

Sodium phytate markedly increased the fecal phosphorus and the urinary phosphorus excretions. Note that during sodium phytate administration the decrease of urinary calcium (to an average value of 6 mg. per day!) and of magnesium are of the same magnitude as the increases in fecal calcium and magnesium. Calcium and phosphorus balances recovered rapidly when sodium phytate was discontinued.

Hydrocortisone increased fecal calcium and slightly decreased urinary calcium excretion. Fecal phosphorus and urinary phosphorus also increased during hydrocortisone and decreased during the brief after-control. The "theoretical" phosphorus balance during hydrocortisone therapy agreed with the "actual" phosphorus balance.

SUMMARY OF CALCIUM BALANCES

In Figure 11 are summarized the average calcium balances of the control periods of these 3

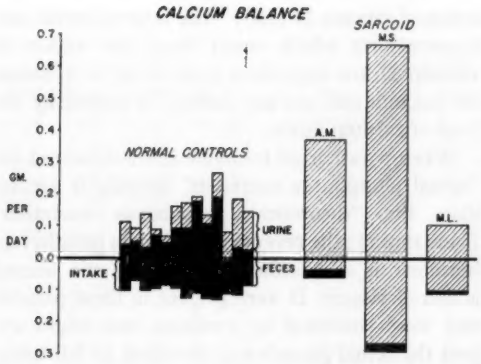


FIG. 11. SUMMARY OF THE CALCIUM BALANCE DATA OF THREE PATIENTS WITH SARCOID IN CONTRAST TO THE CALCIUM BALANCE OF 13 NORMAL SUBJECTS ON A LOW CALCIUM INTAKE

Each column represents the average of one or more six-day collection period.

patients with sarcoid as contrasted with the values for 13 normal controls (17). All these studies were performed on very low and roughly comparable calcium intakes. In each of the patients with sarcoid the fecal calcium was markedly less than normal. In studies 1 and 2 the urinary calcium was greater than could be accounted for by the intake, even with the addition of the 200 mg. per day which normally is excreted in the feces even on very low calcium intakes.

The calcium balance was more closely related to calcium intake in these patients with sarcoid than it is normally.

Cortisone and hydrocortisone increased the fecal calcium and decreased the urinary calcium in studies No. 2 and No. 4. In study No. 3 cortisone normalized the fecal calcium excretion without normalizing the urinary calcium.

Sodium phytate increased the fecal calcium and decreased the urinary calcium. In study No. 3 sodium phytate decreased urinary calcium without markedly altering the already normal fecal calcium excretion.

INTERPRETATION OF THE CALCIUM BALANCE IN SARCOID

The abnormalities of calcium metabolism in these 3 patients with sarcoid are remarkably similar to and suggestive of hypervitaminosis D.

A. Evidence for the first action of vitamin D—increased calcium absorption from the intestine. As shown in Figure 11 all three patients had lower than normal fecal calcium excretions at low levels of calcium intake. Increased calcium absorption would explain this and the clinical observation that most patients with sarcoid do not develop demineralization of the bones despite long-continued hypercalcuria.

Increased calcium absorption has been noted with vitamin D and dihydrotachysterol administration (18) and may be present to some degree in patients with hyperparathyroidism and idiopathic hypercalcuria (19). Although Albright, Bauer, Ropes, and Aub (20) concluded that administered parathyroid hormone was without effect on the fecal calcium yet their studies (21, 22) reveal that patients with hyperparathyroidism may have extremely low fecal calcium values. It may be argued that the low serum phosphorus of hyperparathyroidism favors increased absorption of calcium and phosphorus from the intestine even as it may favor increased resorption of calcium and phosphorus from bone. Against this is a study (22) in which the fecal calcium excretion was slightly less rather than more when the serum phosphorus was raised to normal by means of a huge phosphorus intake in a patient with hyperparathyroidism.

Note that the hypercalcemia and hypercalcuria which might result from increased absorption in sarcoid would be dependent upon and limited in degree by the calcium intake.

TABLE II

Fecal nitrogen, sodium, and potassium values in patients M. S. and M. L.

Patient	Substance	Average control value	Normal values (24)	
			Average	Range
M. S.	nitrogen	0.78 gm./day	1.283	(0.43– 2.30)
	sodium	0.24 mEq./day (0.2% Intake)	3.1	(0.35– 7.68)
M. L.	potassium	6.33 mEq./day (8 % Intake)	9.0	(2.94–17.13)
	sodium	0.82 mEq./day (1.6% Intake)	3.1	(0.35– 7.68)

B. Evidence for the second action of vitamin D—the parathyroid-hormone-like action. In studies No. 1 and No. 3, and initially in study No. 4, the urinary calcium excretion was greater than the calcium intake, a phenomenon unaccounted for by increased calcium absorption alone. The administration of large doses of vitamin D to patients with hypoparathyroidism (18) may increase the urinary phosphorus excretion, decrease the serum phosphorus, raise the serum calcium, and finally raise the urinary calcium to levels greater than the intake. This sequence of events has been epitomized as the parathyroid-hormone-like action of vitamin D (18). The hypercalcemia and hypercalcuria which result from this action of vitamin D are dependent upon bone as a source for calcium and are not limited in degree by the level of calcium intake.

When parathyroid hormone is administered the "actual phosphorus excretion" initially is greater than the "theoretical phosphorus excretion" (vide supra); the reverse is true when parathyroid hormone is discontinued (20). If the second action of vitamin D were present in these patients and were corrected by cortisone one might expect the actual phosphorus excretion to fall below

TABLE III

Complete balance study data for M. L., study 4
(each period is six days)

	Calcium *mg./24°*				Phosphorus *mg./24°*				Nitrogen *Gm./24°*			
Period	Urine	Feces	Intake	Balance	Urine	Feces	Intake	Balance	Urine	Feces	Intake	Balance
1A	301	6	110	−197	743	112	800	− 55	7.92	1.43	11.03	+1.68
1B	310	6	110	−206	774	112	800	− 86	8.44	1.43	11.03	+1.16
2A	244	8	110	−142	740	139	800	− 79	9.42	1.50	11.03	+0.11
2B	207	8	110	−105	772	139	800	−111	9.08	1.50	11.03	+0.45
3A	185	10	110	− 85	730	127	800	− 57	9.15	1.31	11.03	+0.57
3B	137	10	110	− 37	687	127	800	− 14	8.67	1.31	11.03	+1.05
4A	120	12	110	− 22	649	143	800	+ 8	8.05	1.48	11.03	+1.50
4B	120	12	110	− 22	668	143	800	− 11	8.45	1.48	11.03	+1.10
5A	193	160	582	+229	620	158	800	+ 22	8.82	1.38	11.03	+0.83
5B	237	160	582	+185	586	158	800	+ 56	8.30	1.38	11.03	+1.35
5C	226	160	582	+196	593	158	800	+ 49	7.93	1.38	11.03	+1.72
6A	208	155	582	+219	549	131	800	+120	7.81	1.33	11.03	+1.89
6B	198	155	582	+229	654	131	800	+ 15	8.40	1.33	11.03	+1.30
6C	185	155	582	+242	561	131	800	+108	8.84	1.33	11.03	+0.86
7A	12	431	582	+139	1,043	1,064	2,666	+559	8.62	1.72	11.03	+0.69
7B	4	431	582	+147	1,360	1,064	2,666	+242	8.99	1.72	11.03	+0.32
7C	10	431	582	+141	1,244	1,064	2,666	+358	7.53	1.72	11.03	+1.78
8A	5	320	582	+257	1,312	747	2,666	+607	7.92	1.31	11.03	+1.80
8B	2	320	582	+260	1,339	747	2,666	+580	7.76	1.31	11.03	+1.96
8C	2	320	582	+260	1,393	747	2,666	+526	7.77	1.31	11.03	+1.95
9A	2	453	582	+127	1,342	821	2,666	+503	7.33	1.63	11.03	+2.07
9B	5	453	582	+124	1,530	821	2,666	+315	7.72	1.63	11.03	+1.68
9C	8	453	582	+121	1,697	821	2,666	+148	8.75	1.63	11.03	+0.65
10A	125	211	582	+246	934	218	800	−352	9.58	1.31	11.03	+0.14
10B	231	211	582	+140	660	218	800	− 78	8.49	1.31	11.03	+1.23
10C	231	211	578	+136	685	218	800	−103	9.43	1.31	11.03	+0.29
11A	238	249	574	+ 87	599	174	800	+ 27	9.66	1.67	11.03	−0.30
11B	176	249	574	+149	577	174	800	+ 49	9.10	1.67	11.03	+0.26
11C	171	249	574	+154	547	174	800	+ 79	9.43	1.67	11.03	−0.07
12A	137	264	574	+173	549	176	800	+ 75	10.09	1.22	11.03	−0.28
12B	105	264	574	+205	734	176	800	−110	12.19	1.22	11.03	−2.38
12C	97	264	574	+213	741	176	800	−117	12.37	1.22	11.03	−2.56
13A	48	475	574	+ 51	909	309	800	−418	13.77	1.64	11.03	−4.38
13B	127	475	574	− 28	884	309	800	−393	13.41	1.64	11.03	−4.02
13C	104	475	574	− 5	894	309	800	−403	15.24	1.64	11.03	−5.85
14A	139	516	574	− 81	826	363	800	−389	14.33	1.79	11.03	−5.09
14B	115	516	574	− 57	789	363	800	−352	13.32	1.79	11.03	−4.08
14C	95	516	574	− 37	746	363	800	−309	12.58	1.79	11.03	−3.34
15A	235	549	574	−210	475	307	800	+ 18	11.37	2.16	11.03	−2.50
15B	224	549	574	−199	640	307	800	−147	11.72	2.16	11.03	−2.85
15C	166	549	574	−141	530	307	800	− 37	9.27	2.16	11.03	−0.40
15D	164	549	574	−139	485	307	800	+ 8	8.30	2.16	11.03	+0.57
16A	132	496	574	− 54	432	235	800	+133	8.08	1.87	11.03	+1.08
16B	142	496	574	− 64	419	235	800	+146	7.48	1.87	11.03	+1.68
16C	68	496	574	+ 10	423	235	800	+142	6.99	1.87	11.03	+2.17

TABLE III—*Continued*

Potassium *mEq./24°*				Sodium *mEq./24°*				Magnesium *mEq./24°*				Urine chloride *mEq./24°*
Urine	Feces	Intake	Balance	Urine	Feces	Intake	Balance	Urine	Feces	Intake	Balance	
49.78	9.00	62.40	+ 3.62	26.78	0.95	52.10	+ 24.37	6.39	7.37	14.32	+ 0.56	34.31
55.32	9.00	62.40	− 1.92	25.45	0.95	52.10	+ 25.70	7.74	7.37	14.32	− 0.79	26.90
64.02	9.87	62.40	−11.49	36.84	0.85	52.10	+ 14.41	6.30	9.37	14.32	− 1.35	36.64
55.46	9.87	62.40	− 2.93	27.80	0.85	52.10	+ 23.45	6.77	9.37	14.32	− 1.82	28.92
54.65	8.50	62.40	− 0.75	27.99	0.94	52.10	+ 23.17	7.05	8.94	14.32	− 1.67	26.33
52.98	8.50	62.40	+ 0.92	30.88	0.94	52.10	+ 20.28	6.90	8.94	14.32	− 1.52	27.13
56.80	10.10	62.40	− 4.50	36.72	0.50	52.10	+ 14.88	6.65	9.31	14.32	− 1.64	33.19
58.19	10.10	62.40	− 5.89	36.66	0.50	52.10	+ 14.94	7.67	9.31	14.32	− 2.66	33.00
58.80	8.50	62.40	− 4.90	32.91	0.55	52.10	+ 18.64	8.01	10.10	14.32	− 3.79	35.10
54.26	8.50	62.40	− 0.36	28.45	0.55	52.10	+ 23.10	7.62	10.10	14.32	− 3.40	30.88
51.88	8.50	62.40	+ 2.02	32.69	0.55	52.10	+ 18.86	7.76	10.10	14.32	− 3.54	34.62
51.61	8.35	62.40	+ 2.44	35.39	1.15	52.10	+ 15.56	6.43	8.52	14.32	− 0.63	31.90
53.32	8.35	62.40	+ 0.73	31.46	1.15	52.10	+ 19.49	6.72	8.52	14.32	− 0.92	29.80
54.48	8.35	62.40	− 0.43	41.85	1.15	52.10	+ 9.10	7.48	8.52	14.32	− 1.68	33.70
53.30	15.30	62.40	− 6.20	59.74	5.90	141.84	+ 76.20	3.77	13.89	14.32	− 3.34	30.82
44.33	15.30	62.40	+ 2.77	111.62	5.90	141.84	+ 24.32	3.60	13.89	14.32	− 3.17	27.64
39.33	15.30	62.40	+ 7.77	113.36	5.90	141.84	+ 22.58	2.46	13.89	14.32	− 2.03	28.00
45.15	10.83	62.40	+ 6.42	109.40	3.63	141.84	+ 28.81	2.29	11.04	14.32	+ 0.99	37.67
44.56	10.83	62.40	+ 7.01	102.00	3.63	141.84	+ 36.21	2.94	11.04	14.32	+ 0.34	40.18
43.28	10.83	62.40	+ 8.29	119.85	3.63	141.84	+ 18.36	3.23	11.04	14.32	+ 0.05	40.66
45.76	10.57	62.40	+ 6.07	103.92	2.47	141.84	+ 35.45	2.16	16.91	14.32	− 4.75	47.61
50.62	10.57	62.40	+ 1.21	121.86	2.47	141.84	+ 17.51	2.53	16.91	14.32	− 5.12	48.71
48.28	10.57	62.40	+ 3.55	117.38	2.47	141.84	+ 21.99	2.89	16.91	14.32	− 5.48	46.03
56.45	5.00	62.40	+ 0.95	97.52	0.60	52.10	− 46.02	6.82	8.66	14.32	− 1.16	79.74
61.01	5.00	62.40	− 3.61	47.27	0.60	52.10	+ 4.23	7.14	8.66	14.32	− 1.48	67.10
63.58	5.00	62.40	− 6.18	27.66	0.60	52.10	+ 23.84	8.79	8.66	14.32	− 3.13	45.98
61.46	5.27	62.40	− 4.33	25.02	0.80	52.10	+ 26.28	9.72	10.44	14.32	− 5.84	42.35
60.22	5.27	62.40	− 3.09	20.76	0.80	52.10	+ 30.54	8.40	10.44	14.32	− 4.52	39.00
62.28	5.27	62.40	− 5.15	29.58	0.80	52.10	+ 21.72	8.95	10.44	14.32	− 5.07	54.84
75.03	4.47	62.40	−17.10	9.62	0.91	52.10	+ 41.57	12.77	3.40	14.32	− 1.85	25.55
60.10	4.47	62.40	− 2.17	6.59	0.91	52.10	+ 44.60	9.97	3.40	14.32	+ 0.95	19.55
68.65	4.47	62.40	−10.72	8.37	0.91	52.10	+ 42.82	13.59	3.40	14.32	− 2.67	23.26
75.43	8.18	62.40	−21.21	10.30	1.87	52.10	+ 39.93	17.84	7.73	14.32	−11.25	24.32
70.14	8.18	62.40	−15.92	13.41	1.87	52.10	+ 36.82	18.21	7.73	14.32	−11.82	29.82
73.23	8.18	62.40	−19.01	17.76	1.87	52.10	+ 32.47	15.62	7.73	14.32	− 9.03	29.39
77.22	9.85	62.40	−24.67	17.28	2.20	52.10	+ 32.62	22.49	11.04	14.32	−19.21	48.00
64.31	9.85	62.40	−11.76	15.23	2.20	52.10	+ 34.67	15.62	11.04	14.32	−12.34	34.84
65.67	9.85	62.40	−13.12	17.44	2.20	52.10	+ 32.46	17.40	11.04	14.32	−14.12	36.46
48.78	7.17	62.40	+ 6.45	89.01	4.63	52.10	− 41.54	15.79	12.37	14.32	−13.84	96.09
60.31	7.17	62.40	− 5.08	251.05	4.63	52.10	−203.58	16.83	12.37	14.32	−14.88	208.78
52.85	7.17	62.40	+ 2.38	125.96	4.63	52.10	− 78.49	14.28	12.37	14.32	−12.33	110.94
57.27	7.17	62.40	− 2.04	63.14	4.63	52.10	− 15.67	13.75	12.37	14.32	−11.80	84.23
58.32	6.03	62.40	− 1.95	44.83	2.13	52.10	+ 5.14	11.94	10.41	14.32	− 8.03	66.74
59.67	6.03	62.40	− 3.30	28.23	2.13	52.10	+ 21.74	14.55	10.41	14.32	−10.64	53.87
57.87	6.03	62.40	− 1.50	28.60	2.13	52.10	+ 21.37	6.25	10.41	14.32	− 2.34	46.46

the "theoretical" phosphorus excretion when cortisone or hydrocortisone therapy was begun. In fact the "theoretical phosphorus balance," as indicated by the dotted lines on the phosphorus balances in Figures 1 and 8, agrees well with the "actual phosphorus balance." It has been noted in other studies (23) that cortisone and hydrocortisone may increase the actual phosphorus excretion above the "theoretical" phosphorus excretion initially, an effect opposite to the one anticipated in this study and one which might explain the failure of the "theoretical" phosphorus to deviate from the actual phosphorus balance in these studies. A parallel phenomenon, potassium diuresis in excess of that predicted from the nitrogen balance, was observed at the start of cortisone and hydrocortisone therapy in studies No. 2 and No. 4.

In the second study on patient M. S., fecal calcium was greater than the intake which suggests that this dose of cortisone had corrected the first action of vitamin D. However, the urinary calcium remained high, showing that the two suspected actions of vitamin D, if present in these patients, are separable and not necessarily related. It is suggested that sodium phytate decreased the urinary calcium in study No. 3, not by decreasing calcium absorption, but by supplying the inorganic phosphorus for the phosphorus-diuretic activity

present, thus sparing the bones from increased resorption and thereby decreasing the serum calcium.

C. Evidence for a third action of vitamin D—increased absorption of nitrogen, sodium, potassium, magnesium, and iron. In Table II are summarized some control fecal values of the sarcoid patients as contrasted with normal values.

These fecal values of the sarcoid patients are consistently though not dramatically less than normal.

In Figures 12 and 13 attention is called to the lower values noted for fecal nitrogen, magnesium, sodium, and potassium at the beginning of the study (days 0 to 4) and at the height of AT-10 (dihydrotachysterol) action (days 36 to 40) than those noted in the rest of the study. These examples of increased absorption coincided with

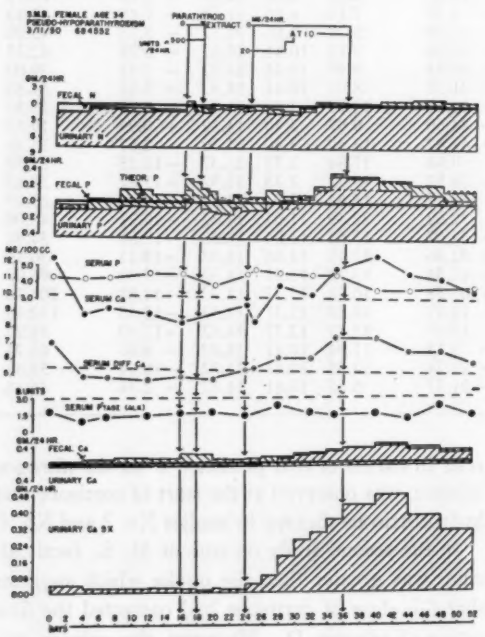


FIG. 12. THE EFFECT OF PARATHYROID EXTRACT AND AT-10 (DIHYDROTACHYSTEROL) ON THE NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES OF A PATIENT WITH PSEUDOHYPOPARATHYROIDISM

The patient received sufficient AT-10 immediately before and again during this study to cause moderate hypercalcemia.

By the serum diffusible calcium is meant that amount passing a cellophane filter under pressure. These measurements were made by Dr. John Dawson.

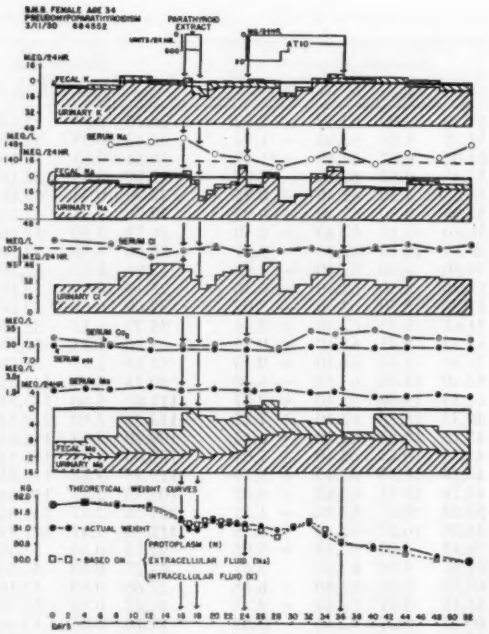


FIG. 13. THE EFFECT OF PARATHYROID EXTRACT AND AT-10 ON THE POTASSIUM, SODIUM AND MAGNESIUM BALANCES AND URINARY CHLORIDE OF A PATIENT WITH PSEUDOHYPOPARATHYROIDISM

Note that minimum fecal values occurred during days 0 to 4 and 36 to 40 and coincided with the episodes of maximum hypercalcemia.

hypercalcemia produced by large doses of AT-10, a vitamin D congener, just before the study began and again during the study. Vitamin D and AT-10 are closely related compounds and have markedly similar effects in man (25).

Since the initial high serum iron values in patient M. L. could not be demonstrated a second time there is not adequate evidence for increased iron absorption in this condition. It would seem that increased absorption of many substances may occur in sarcoid. It remains for future studies to determine whether iron, a substance normally poorly absorbed, is also better absorbed in sarcoid.

DISCUSSION

These studies strongly suggest, though they do not prove, that the hypercalcuria of sarcoid is due to endogenous hypervitaminosis D. If this is so, are t..ese patients photosensitive in such a way that their skin forms excessive vitamin D on nor-

mal actinic exposure? Are they unable to inactivate vitamin D? Does the sarcoid tissue form a vitamin D-like substance? Is the intestinal flora in sarcoid capable of forming vitamin D-like substances? In favor of the first suggestion is the dry pigmented skin noted generally in M. S. and on the face in patient M. L. Both these patients have recently had recrudescence of hypercalcemia in the summer. In favor of the second suggestion is the observation by Larsson, Liljestrand, and Wahlund (26) of hypercalcemia in 12 of 24 patients with sarcoid treated with 30,000 to 140,000 units vitamin D daily for 6 to 124 days. Non-sarcoid subjects are rarely poisoned with less than 200,000 units administered for months to years. We know of no observations pertinent to the other two possibilities and there are undoubtedly other possible explanations for the changes noted.

The mode of action of ACTH, cortisone and hydrocortisone in sarcoid is obscure. It is known that these drugs may temporarily or permanently cause the disappearance of sarcoid granulomata (27). Since vitamin D and cortisone possess similar structural formulae there may be a direct antagonism between these substances. However, in our experience (24) patients with Cushing's syndrome do not have unusually high fecal calcium excretion nor does cortisone administration increase fecal calcium except in patients with sarcoid.

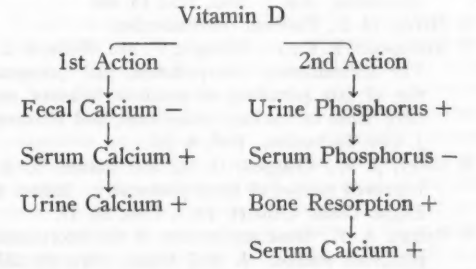
Dent, Flynn, and Nabarro (28) in 1953 demonstrated correction by cortisone of hypercalcemia and depressed renal function in patients with sarcoid. They concluded that bone destruction was the cause of the hypercalcemia. More recently Anderson, Harper, Dent, and Philpot (29) have concluded that increased calcium absorption is the cause of hypercalcemia in sarcoid. Lack of published standards for normal calcium balance on calcium intakes of 800 to 1,000 mg. daily and lack of control data after cortisone administration weaken their evidence for increased calcium absorption in these later studies. It is of interest that most of the instances of increased symptoms due to sarcoid in Dent's patients were reported to occur in the summer months.

SUMMARY AND CONCLUSIONS

Four complete balance studies on three patients with sarcoid demonstrate the following abnormalities of calcium metabolism:

1. Increased calcium absorption from the intestine.
2. Hypercalcemia.
3. Increased calcium excretion in the urine.
4. Urinary calcium excretion greater than can be accounted for by increased absorption alone.

These changes strongly suggest endogenous vitamin D intoxication, in which the following sequence of events is observed:



Attention is called to evidence for a possible third action of vitamin D . . . the property of increasing intestinal absorption over a wide spectrum of substances. Thus, increased absorption of nitrogen, sodium, potassium and magnesium was noted in one of these studies and in a patient with pseudohypoparathyroidism in whom hypercalcemia was produced by excessive doses of AT-10, a vitamin D congener.

Cortisone increased the fecal calcium and decreased the urinary calcium excretion of two patients. The mode of action of cortisone in this disease is not known. Sodium phytate also decreased urinary calcium excretion, apparently primarily by increasing fecal calcium excretion.

Sodium phytate appears to be the treatment of choice of the hypercalcuria of sarcoid and its use is suggested for other instances of increased calcium absorption such as vitamin D poisoning, idiopathic hypercalcuria, and idiopathic hypercalcemia of infants (30).

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CAROTENOIDS IN MAN. THE DISTRIBUTION OF EPIPHASIC CAROTENOIDS IN ATHEROSCLEROTIC LESIONS [1]

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Carotenoid pigments are yellow and red colored lipids widely distributed throughout nature. Those most commonly encountered contain 40 carbon atoms and have structures closely related to that of lycopene (Figure 1). Approximately 80 carotenoid pigments are known, each with a characteristic absorption spectrum. In some, closed rings are present at the ends of the molecule, while others contain one or more moles of oxygen in carbonyl or hydroxyl groups. Carotenoids containing no hydroxyl groups are called epiphasic carotenoids because when equilibrated between two immiscible solvents (petroleum ether and aqueous 90 per cent methanol), they pass completely into the petroleum ether epiphase.

Certain carotenoids (chiefly epiphasic) can be converted to Vitamin A, and their role in human nutrition has been extensively studied (1). Carotenoids have long been known to contribute to the yellow color of human depot fat, adrenal cortex, and atheromatous plaques (2), but this phenomenon has attracted little attention. Since studies of carotenoids as provitamins A have demonstrated that they cannot be synthesized by man, their occurrence in atheromatous plaques provides evidence that a dietary lipid may be deposited in the lesions of this disease. Accordingly, the concentrations of total epiphasic carotenoids and total cholesterol were studied in thirty human aortas having varying degrees of atherosclerosis. The findings indicate that atherosclerotic lesions of the aorta accumulate carotenoids in amounts related to the age and extent of the lesions.

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METHODS

1. *Collection of specimens.* The aortae were obtained without selection from autopsies performed on patients ranging in age from 10 to 90 years. After removal from the body the aortae were stored at 4 degrees centigrade under a moist towel for periods up to 48 hours before examination and dissection. An arbitrarily chosen standard segment 3 inches long, which included the entire width of the descending arch just caudal to the left subclavian artery, was studied in all cases. In 12 patients, 2 additional segments were studied. These latter specimens also included the entire width of the aorta and were 3 inches long. They were measured cephalad from the coeliac axis and the bifurcation of the aorta respectively. Segments were cut to measure and then stripped of adventitia in preparation for grading.

2. *Grading of atherosis.* The segments were graded for extent and character of atherosclerosis in the following manner:

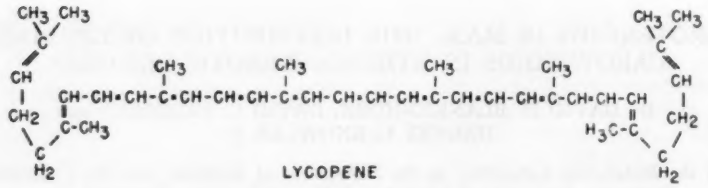
Extent: The extent to which the atherosis involved the intima was graded 1 to 4, depending on whether 1 to 25 per cent, 25 to 50 per cent, 50 to 75 per cent or 75 to 100 per cent of the intimal surface of the segment appeared to be covered by plaques.

Character: The character of the most representative plaque was assigned a grade of 1 to 4 according to a descriptive scheme based on the assumption that the morphology of atherosclerotic plaques changes in a serial manner. The characteristics graded are in all probability related to the age of the lesions. However, the rate at which atherosclerotic lesions undergo change is uncertain. Moreover, it is unknown whether all lesions change at the same rate. Consequently, only relative age can be judged.

The criteria used for assigning a grade are outlined below:

Grade 1. Gross: Visible intimal thickening and gelatinous non-yellow translucent discrete plaques. *Microscopic:* Thickening and edema of the intima, without distinct plaque formation.

Grade 2. Gross: Yellow, opaque plaques without evidence of calcium present. *Microscopic:* Well-defined lipid laden plaques, the lipid being primarily contained within macrophages. Moderate amounts of extracellular cholesterol and minimal intimal fibrosis overlying the lipid-



LYCOPENE

FIGURE 1

containing areas were considered compatible with this grade.

Grade 3. Gross: Thick pale gray-white lesions, often confluent, with evidence of fibrosis and calcification present. *Microscopic:* Plaques with lipid predominantly extracellular and with a significant intimal fibrosis over the lipid mass. Few lipid containing macrophages and minimal calcification and ulceration present.

Grade 4. Gross: Changes more severe than grade 3, including ulceration, calcification or medial involvement. *Microscopic:* Lesions showing more severe changes than seen in lower grades, including increased fibrosis, calcification, ulceration and almost completely extracellular lipid.

This scheme for grading atherosclerosis deserves special mention. It represents an attempt to classify the morphology of atherosclerosis so that the results of chemical assay may be placed in the perspective of pathologic anatomy. In this scheme two attributes of the lesion are estimated, their extent and their character or relative age. The severity of atherosclerosis is considered to be a function of these two attributes.

To test the validity of this plan of grading, the specimens were assayed for total cholesterol since cholesterol is the major lipid found in atherosclerotic plaques (3–6), and because previous workers have shown that the amount of atherosclerosis found in the aorta is proportional to its cholesterol content (5, 6). To minimize the effect of individual variation in size, thickness and water content of the aorta, the data have been expressed in milligrams of cholesterol per square centimeter of intimal surface as suggested by McMillan, Horlick, and Duff (7). In Figure 2 the individual cholesterol content per square centimeter of each of 30 segments of thoracic aorta is plotted against the grade assigned to that segment. The grades of extent and relative age are plotted separately, followed by a plot of the average of these two grades. A significant relation ($t = 6.2$) is demonstrated between the average grade and cholesterol content per square centimeter. Accordingly, it is believed that this grading scheme is a valid basis for the rough quantitation of the severity of atherosclerosis. Severity, as used hereafter, is defined as a term for semi-quantitative description of atherosclerosis which is estimated by averaging two numerical grades, one of which indicates the extent of the lesion, the other its relative age or character.

In its present state this grading scheme is considered to be only semi-quantitative. However, further refinement and application of this technique may yield information of value. In Figure 2 the first two plots shown demonstrate that there is no significant difference ($p > 0.5$) between the relation of the cholesterol content per square centimeter and the extent and relative age of the lesions. The segments of mid-abdominal and lower abdominal aorta studied also showed this finding. It would be of interest to know to what extent this relation applies to the remainder of the vascular tree in man.

When segments are graded grossly the judgment of the

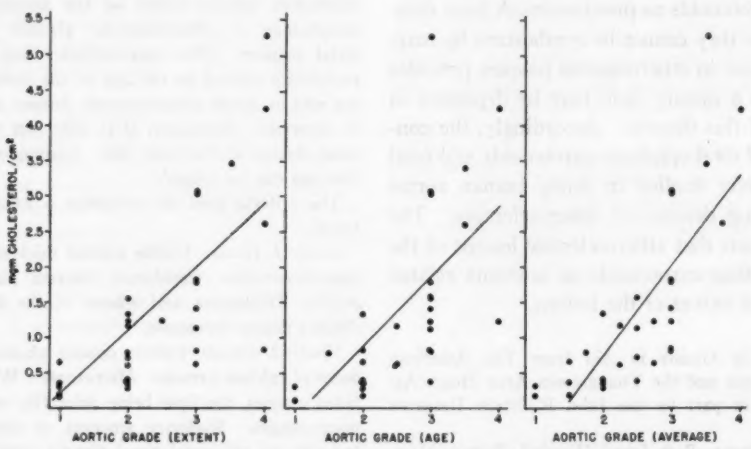


FIG. 2. THE CHOLESTEROL CONTENT OF THE HUMAN AORTA

examiner as to the character of the most common plaque may be influenced by the degree to which the intimal surface is covered by atheroma. However, this error is excluded when grading is done microscopically. When 16 plaques from different aortas in this series were graded both grossly and microscopically, a satisfactory agreement between the estimates was found. Thirteen plaques were placed in the same group, two were graded a group too high grossly and one a group too low.

3. Preparation of tissue for assay. After gross grading, several radial cuts were made in the specimen to make it lie flat, then it was placed on Whatman No. 50 filter paper and traced. Later the area of each segment was determined to the nearest 0.1 cm., with a polar planimeter. Pressed between two filter papers, the specimen was dried for 48 hours at approximately 0.5 cm. mercury vacuum over $CaSO_4$ at room temperature, and then weighed. It was next ground to a twenty mesh powder in an intermediate model Wiley Mill, and the powder brought to constant weight over $CaSO_4$ for assay of carotenoid and cholesterol content.

4. Analytical methods. Total epiphasic carotenoids and total cholesterol were determined using a combination of several preexisting techniques for carotenoid and cholesterol analysis in serum (8–11). Approximately 250-mg. aliquots of powdered aorta were washed with 3.0 cc. of water into red, glass-stoppered cylinders. After addition of 3.0 cc. of freshly made 1.0 N KOH in 90 per cent ethanol, the cylinders were loosely stoppered, heated to their necks in a water bath at 50° to 60° C. for 30 minutes, and shaken vigorously at 10-minute intervals. The cylinders were then allowed to cool to room temperature, 6.0 cc. of Hexane (Skelly-solve B) were added, and the stoppers tightly sealed with a small drop of mineral oil. The cylinders were rotated end over end mechanically for 10 minutes, and allowed to stand for 10 minutes. Four cc. of the upper phase were transferred to a 16×100 mm. optically matched test tube cuvette and tightly stoppered. Optical density was measured in a Beckman DU spectrophotometer with test tube attachment at 440 mu., employing a blue sensitive phototube and 0.14 slit. For cholesterol determination an aliquot of the Hexane phase was transferred to a second matched test tube cuvette and dried at water vacuum. Two cc. of glacial acetic acid were added to each tube and heated with stirring in boiling water for 15 seconds. The procedure from this point forward was that of Sperry and Webb (11). A cholesterol standard, prepared in the manner of Abell, Levy, Brodie, and Kendall (10) and carried through the entire procedure was used as a solvent blank for the carotenoid determination and to supply an internal standard for the cholesterol determination.

All solvents used were reagent grade, except ethanol which was USP, and all were redistilled. Test tubes (A. H. Thomas and Co. No. 9446) used as optical cuvettes were matched to a 0.3 per cent transmission using 0.7 millimolar aqueous potassium dichromate against an air blank. Carotenoid content was calculated using the molecular extinction $E'_{440} = 231 \times 10^3$ which was determined experimentally for this instrument using a mixture of Alpha and Beta Carotene. The published values of E'_{440} for the major epiphasic carotenoids known to be present in human tissue are: Alpha Carotene $E'_{440} = 310 \times 10^3$, Beta Carotene $E'_{440} = 330 \times 10^3$, Lycopene $E'_{440} = 210 \times 10^3$ (12). Bile pigments do not interfere with the determination (13).

Fifty-two samples from 30 patients were analyzed. All analyses were carried out in duplicate or triplicate as indicated. Duplicate analyses of aorta revealed average deviations from the mean of ± 3.3 per cent for cholesterol, ± 5.0 per cent for carotenoids when the aortic carotenoid content was in excess of .05 μg. per cm.^{2} and ± 12 per cent when the content was less than this amount. When the range of values exceeded 5 per cent of the mean, triplicate or quadruplicate determinations were made if sufficient material was available. Recoveries of added carotenoid to aortic powder ranged from 88 per cent to 93 per cent regardless of whether the initial content was low or high, respectively. The high range of analytical error found in samples with low carotenoid content resulted from production of insufficient color to stay in the effective range of the instrument (14). Nevertheless, the magnitude of change encountered in different samples was so great that error in duplication was insignificant in final evaluation of data.

RESULTS AND DISCUSSION

In Table I are given the carotenoid and cholesterol contents of the aortas studied. The findings are listed serially by severity of atherosclerosis, and by cholesterol content within each grade of severity, as the values did not appear to be influenced by the area from which the segments were taken, or by the age, sex, or state of nutrition of the patient.

The data given in Table I have been divided into serial quarters and the average severity, average carotenoid content, average cholesterol content, average carotenoid-cholesterol ratio of each quarter are shown in Table II. With increase in the severity of the lesions, there were accompanying significant increases in carotenoid and cholesterol contents. However, the small increase in carotenoid-cholesterol ratio was not significant ($p > 0.1$).

The relative constancy of the carotenoid-cholesterol ratio is worthy of comment. Cholesterol is the major lipid present in atherosclerosis (3–6), but it is not known whether it accumulates from outside the plaques or is formed *in situ*. It is generally accepted that carotenoids are derived from exogenous sources. In man, dietary depletion of these pigments decreases their blood and

TABLE I

Total epiphasic carotenoid and cholesterol content of the human aorta

Severity of atherosclerosis*	Cholesterol mg./cm.²	Carotenoids μg./cm.²	Ratio†	Aortic zone‡	Age and sex§	Final state of nutrition‖
4	8.76	1.92	.22	MA	67M (5)	WN
4	5.25	.46	.09	LA	78M (1)	E
4	4.40	.46	.10	LA	67M (5)	WN
4	4.25	.79	.19	Th	77M (20)	WN
4	2.79	.48	.17	LA	67M (6)	WN
4	1.97	.17	.09	LA	61F (24)	E
3 3/4	4.05	.57	.14	LA	64M (2)	PN
3 3/4	2.62	.37	.14	Th	56F (29)	PN
3 1/2	5.29	1.18	.22	Th	60F (30)	WN
3 1/2	3.47	.60	.17	Th	76F (14)	WN
3 1/2	1.14	.11	.10	MA	61F (24)	E
3	3.86	.23	.06	MA	78M (1)	E
3	3.07	1.12	.35	Th	54F (13)	WN
3	3.04	.53	.17	Th	54F (21)	WN
3	2.44	.48	.20	LA	65M (22)	WN
3	1.83	.28	.15	LA	57M (8)	WN
3	1.82	.34	.19	Th	67F (6)	WN
3	1.79	.25	.14	Th	61F (24)	E
3	1.41	.29	.20	Th	67M (15)	WN
3¶	1.23	.04	.03	Th	79M (19)	E
3	1.06	.29	.29	MA	67F (6)	WN
3	.84	.09	.10	Th	50M (4)	PN
3	.82	.11	.15	Th	49M (17)	WN
3	.81	.12	.15	LA	59F (3)	WN
2 3/4	1.24	.19	.15	Th	80F (26)	WN
2 3/4	.63	.06	.10	Th	61F (12)	WN
2 1/2	1.56	.07	.04	Th	78M (1)	E
2 1/2	1.13	.08	.07	Th	50M (27)	WN
2 1/2	.71	.08	.12	Th	63M (18)	WN
2 1/2	.39	.07	.18	Th	71M (23)	PN
2 1/4	1.17	.13	.11	Th	73M (9)	PN
2 1/4	.62	.04	.06	Th	64M (2)	PN
2	1.34	.20	.15	Th	67M (5)	WN
2	1.03	.18	.18	MA	59F (3)	WN
2	.70	.11	.16	MA	71M (23)	PN
2	.55	.09	.16	MA	65M (22)	WN
2	.39	.08	.21	Th	54F (16)	WN
2	.39	.05	.14	LA	71M (23)	PN
2	.35	.08	.24	Th	52M (11)	E
2	.32	.03	.09	MA	50M (4)	PN
2	.28	.05	.18	Th	65M (22)	WN
1 3/4	.79	.11	.14	Th	59F (3)	WN
1 3/4	.45	.03	.07	MA	64M (2)	PN
1 1/2	.66	.09	.14	Th	57M (8)	WN
1 1/2	.35	.03	.09	Th	51M (10)	WN
1 1/2	.22	.02	.11	LA	50M (4)	PN
1 1/2	.18	.01	.06	Th	38F (7)	E
1 1/2	.12	.01	.05	MA	38F (7)	E
1 1/2	.11	.02	.18	LA	38F (7)	E
1	.31	.03	.09	Th	42M (25)	WN
1	.23	.03	.14	LA	32F (28)	WN
1	.18	.03	.16	MA	32F (28)	WN
1	.18	.03	.16	Th	32F (28)	WN

* Severity of atherosclerosis is estimated by averaging two grades, one of which indicates the extent of atherosclerotic lesions, the other their character.

† Ratio is the ratio of carotenoids in micrograms to cholesterol in milligrams.

‡ Aortic zone: Th, Thoracic Aorta; MA, Midabdominal Aorta; LA, Lower Abdominal Aorta.

§ The number in parentheses is a code number assigned to each patient; 12 patients had more than one area of the aorta examined.

‖ State of nutrition: WN, Well Nourished; PN, Poorly Nourished; E, Emaciated.

¶ This aorta had extensive medical calcification with involvement of the adventitia in addition to atherosclerosis.

TABLE II

Quarterly average values, data from Table I

Quarter	Severity of atherosclerosis	Total epiphasic carotenoids $\mu g./cm.^2$	Total cholesterol $mg./cm.^2$	Carotenoids $\mu g.$ / Cholesterol $mg.$
1	4	.65	3.9	.17
2	3	.24	1.4	.16
3	2	.10	.71	.14
4	1 1/2	.04	.31	.13

tissue concentrations to negligible amounts (15). In addition, the signs of Vitamin A deficiency produced can be promptly relieved by administration of carotenoids (15). The present findings therefore give evidence that one dietary lipid not only is present in atherosclerotic plaques but accumulates in direct proportion to age and extent of the lesions.

It is unknown whether carotenoid pigments are concerned with the production of atherosclerotic plaques or if their role is of a secondary nature. It has been suggested by earlier investigators that the presence of carotenoids in atherosclerosis stems from the solubility of these pigments in the other lipids present (2). Although this may be the case, there is evidence that other factors are of more importance in determining their distribution. Some areas high in lipid contain only negligible concentrations of carotenoids (16). The electrophoresis of serum yields a distribution of carotenoids different from total lipid or any demonstrable individual lipid (17). If the solubility of carotenoids in other lipids were to determine their distribution, they would be carried with the lipids in which they are dissolved and show the same mobility. However, this is not the case, and their migration is more suggestive of an affinity for certain proteins (18).

Further studies are indicated on the mechanism of carotenoid accumulation in atherosclerotic plaques. The use of these pigments may prove to be a useful aid in the study of atherosclerosis.

SUMMARY

Studies have been made on the concentrations of carotenoids and cholesterol in human atherosclerotic lesions. The findings indicate that these substances increase in concentration with extent and relative age of lesions. The carotenoid-cholesterol ratio is not significantly changed. Hence, one lipid of exogenous origin is shown to accumulate in plaques in direct proportion to the severity of atherosclerosis.

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STUDIES OF THE ANEMIA AND IRON METABOLISM IN CANCER [1]

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Anemia is a common occurrence in patients with malignant neoplastic disease. It is often associated with chronic blood loss and occasionally with chronic infection. However, in the majority of instances, the anemia cannot be ascribed to these causes and no basis for its development is discovered other than the existence of cancer. It has been interpreted by some investigators as a reflection of toxic bone marrow depression or destruction of the marrow by tumor metastases. However, hemoglobin concentration and the size of the total erythron (1–3) may be normal in the presence of bone marrow metastases. Recently it has been proposed (4–6) that the anemia of cancer may occasionally result from a diminution in the life span of the red cell. Also, an increased rate of red cell destruction has been noted in leukemia and lymphoma (7). Hypoferremia in cancer in the absence of chronic blood loss has been described by many investigators (8–10). Usually, but not invariably, this has been associated with anemia. Its pathogenesis and its relationship to the anemia of cancer are alike obscure.

The purpose of this investigation has been to study the effect of cancer on iron metabolism and to delineate, if possible, the mechanisms responsible for anemia in patients with neoplastic diseases.

METHODS

I. *Clinical Material*

Thirty-eight patients with a diagnosis of carcinoma except for patients No. 17 and 20 were studied. Metastases were present in all but 4 of these patients (Tables I and II). Primary renal and liver disease, blood loss, overt hemolytic anemia, and infection were excluded by clinical examination and laboratory findings.

II. *Experimental Plan*

The subjects were hospitalized during the initial period of investigation, and subsequently when necessary. Fecal urobilinogen and radioiron studies were begun during the first week of hospitalization. A transfusion of labelled red cells for the purpose of measuring erythrocyte survival was begun after completion of the above studies. All phases of this program were completed in only 3 cases.

III. *Procedures*

1) *Hematological*

Erythrocyte count, hematocrit (11), and hemoglobin (12) were measured in all patients. Reticulocyte count by the aqueous cresyl-blue method, quantitative serum bilirubin (13) and Coombs test were done on most of the patients.

2) *Urobilinogen excretion and hemolytic indices*

Fecal urobilinogen was determined on four-day stool collections by the method of Schwartz, Sborov, and Watson (14) and the hemolytic index then calculated (15).

3) *Blood volume determinations*

The red blood cell volume was determined in 15 patients by the P^{32} tagged red cells technique, and in the remaining patients, plasma volume was determined by the Evans blue dye method of Gibson and Evans (16). When the total red cell volume was calculated from the Evans blue determined plasma volume, the venous hematocrit was corrected by the factor of 0.92 (17).

4) *Erythrocyte survival studies*

(a) *Ashby red cell survival studies.* Thirteen individual red cell survival studies were performed in 12 patients. In patients No. 23, 27 and 31, x-ray treatment ranging from 2,000 to 8,000 r had been completed within a minimum of 16 days before survival studies began. Each patient was transfused with 500 or 1,000 cc. of normal donor blood, freshly collected in ACD, except in three cases, where the blood was from 3 to 6 days old. Inagglutinable counts were 20,000 or below per $mm.^3$ in all but two cases. The differential agglutination tech-

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TABLE I

Clinical, hematological, and radioiron data on 38 cancer patients

Patient	Diagnosis	Hct. %	Hgb. gm. %	MCHC†	Reticulocyte count %	Fecal urobilinogen mg./day	Hemolytic index	Serum iron γ/100 cc.	Plasma vol. cc.	Rbc vol. cc.	Fe^{59} $T_{\frac{1}{2}}$ hr.	Plasma iron turnover mg./day	Plasma iron turnover mg./hr./L. rbc	Rbc utilization of Fe^{59} %	Rbc iron turnover mg./day	Rbc iron turnover mg./hr./L. rbc	Rbc renewal rate %
1	Cancer of prostate. Metastatic to lumbosacral spine.	19.0	6.2	32.7	2.7			58.0	2,900	615	0.42	66.7	4.520	100	66.7	4.520	10.8
2	Cancer unknown primary site. Metastatic to pelvic bones.	41.0	14.3	34.8	1.2			78.0	2,636	1,595	1.35	25.7	0.671	98	25.7	0.671	1.6
3	Resected cancer of the breast. Metastatic to lung and pleura.	40.0	13.9	34.7	1.0	40.0	8.0	38.8	2,393	1,400	0.58	26.6	0.786	93	24.0	0.714	1.7
4	Cancer of breast. Metastatic to liver, ribs, spine (on androgen Rx).	29.5	9.0	29.5	4.2			71.0	3,800	859	1.05	45.0	2.189	69	31.5	1.525	2.1
5	Resected cancer of breast. Metastatic to lung and brain.	26.5	8.5	32.0	1.7	75.0	29.0	62.0	2,200	710	1.03	22.0	1.127	60	13.2	0.775	2.0
6	Resected cancer of breast. Metastatic to spine.	34.5	11.0	31.9				56.0	3,400	1,578	1.27	25.0	0.690				
7	Cancer of kidney. Metastatic to lung, liver, kidney.	36.8	12.2	33.2	1.8	40.0	10.0	34.5	3,185	1,750	0.40	45.7	1.708	90	41.1	0.936	2.3
8	Resected cancer of breast. Metastatic to ribs, spine, lungs (on cortisone Rx).	33.0	9.8	30.0	4.5		37.0	116.0	2,278	990	1.29	34.0	1.434	65	22.1	0.930	2.2
9	Cancer of lung. Metastatic to brain.	41.0	14.1	34.0		118.0		129.0	3,000	1,815	1.08	59.0	1.355	100	59.0	1.355	3.3
10	Cancer of lung. No metastases.	44.0	14.0	31.8	1.5			35.0	2,018	1,200	0.66	32.0	1.108	82	26.2	0.910	1.2
11	Cancer of lung. Metastatic to pleura, nodes.	48.0	16.7	34.8				62.5	2,757	2,021	0.98	29.2	0.604	100	29.2	0.604	1.5
12a	Resected cancer of breast. Metastatic to orbit.	42.0	14.2	33.8				98.0	2,018	1,200	1.65	19.9	0.692	90	17.8	0.618	1.5
12b 76 days later	As above. Metastatic to bone, lung and skin.	36.7	11.5	31.4	1.0			34.6	2,010	1,011	0.57	15.1	0.623	100	15.1	0.623	1.6

* P^{32} determined red cell and plasma volume. Evans blue used in all other patients.
† Mean corpuscular hemoglobin concentration—gm. Hgb. per 100 cc. of packed red cells.

TABLE I—*Continued*

Patient	Diagnosis	Hct. %	Hgb. gm. %	MCHC†	Reticulocyte count %	Fecal urobilinogen mg./day	Hemolytic index	Serum iron γ/100 cc.	Plasma vol. cc.	Rbc vol. cc.	Fe^{59} $T_{\frac{1}{2}}$ hr.	Plasma iron turnover mg./day	Plasma iron turnover mg./hr./L. rbc	Rbc utilization of Fe^{59} %	Rbc iron turnover mg./day	Rbc iron turnover mg./hr./L. rbc	Rbc renewal rate %
13	Resected cancer of breast. Metastatic to axillary nodes.	41.0	13.5	32.6				98.0	2,270	1,374	1.68	22.0	0.667	95	21.1	0.640	1.5
14	Cancer of lung. Metastatic to pleura.	42.0	13.0	31.1				53.5	2,037	1,281	0.70	25.9	0.843	77 (at 5 days)	19.9	0.768	1.6
15	Cancer of breast. Metastatic to local nodes.	34.0	11.7	34.4	3.0			60.0	1,860	848	0.83	22.0	1.085	89	19.6	0.963	2.3
16	Resected cancer of colon. Metastatic to liver.	38.8	13.0	33.5				80.0	2,700	1,499	1.73	21.0	0.580	92	19.2	0.534	1.3
17	Lymphosarcoma of liver, spleen and bone marrow.	25.5	8.2	32.2	0.5	132.0	38.0	165.5	3,800	859	2.25	46.7	2.270	5	2.4	0.114	0.3
18	Cancer of lungs. Metastatic to brain.	43.2	14.7	34.0				55.0	1,941	1,278	1.06	18.0	0.587	96	17.5	0.570	1.4
19	Cancer of lung. No metastases.	45.0	14.9	33.0				79.0	3,810	2,692	1.30	39.3	0.608	96	37.3	0.518	1.1
20	Fibromyxosarcoma of thigh, back, lungs.	39.0	13.8	35.0	1.0	30.0	8.0	75.0	2,593	1,451	2.00	16.2	0.465	100	16.2	0.465	1.1
21a	Cancer prostate. Metastatic to lumbosacral spine, pelvis.	31.0	9.1	30.0	1.1			86.8	*2,833	1,121	1.10	37.2	1.383	100	37.2	1.379	3.3
21b 8 mos. later	As above (on estrogen Rx).	27.2	8.1	30.0		50.0	14.0	45.0	*2,845	1,486	1.10	19.3	0.505	100	18.1	0.505	1.3
22	Cancer of kidneys. Metastatic to lung, spine.	29.0	8.8	30.6		77.0	19.7	37.5	*3,097	1,282	0.70	27.6	0.897	100	27.6	0.897	2.2
23	Cancer of lung. Metastatic to scapula.	30.5	9.0	29.5				44.1	*2,168	950	0.38	41.8	1.833	88	38.7	1.698	3.8
24	Cancer of prostate. Metastatic to spine, pelvis.	33.6	10.5	31.4				39.7	*2,248	1,137	0.85	17.5	0.641	90	16.0	0.641	1.4
25	Cancer of unknown primary site. Metastatic to axillary nodes.	40.3	13.5	33.5	0.3			104.0	*2,492	1,672	1.00	43.8	1.068	100	43.0	1.068	2.5
26	Resected cancer of stomach. Metastatic to liver, abdominal nodes.	35.1	10.2	30.0				80.9	*2,463	1,332	0.62	47.8	1.495	100	47.8	1.495	3.5
27	Cancer of lung. Metastatic to nodes, liver.	28.1	8.5	30.3		150.0	32.8	49.3	*3,829	1,494	0.95	89.7	2.502	100	89.7	2.502	5.8

TABLE I—*Continued*

Patient	Diagnosis	Hct. %	Hgb. gm. %	MCHC†	Reticulocyte count %	Fecal urobilinogen mg./day	Hemolytic index	Serum iron γ/100 cc.	Plasma vol. cc.	Rbc vol. cc.	Fe^{59} $T_{\frac{1}{2}}$ hr.	Plasma iron turnover mg./day	Plasma iron turnover mg./hr./L. rbc	Rbc utilization of Fe^{59} %	Rbc iron turnover mg./day	Rbc iron turnover mg./hr./L. rbc	Rbc renewal rate %
28	Resected cancer of breast. Metastatic to ribs, spine, skin.	36.1	11.3	31.3	2.2	65.0	17.0	86.4	*2,115	1,193	1.10	27.6	0.964	77	26.2	0.915	2.1
29a	Resected cancer of breast. Metastatic to humerus, ribs, spine.	34.3	10.7	31.3		34.0	12.0	68.8	*1,713	893	1.05	18.7	0.873	100	18.7	0.840	2.1
29b 4 mos. later	As above.	26.5	7.9	29.9	2.3			58.0	*2,213	742	0.85	25.1	1.409	70	17.5	0.986	2.3
29c 11 mos. later	New bone metastases (on cortisone Rx).	28.0	9.2	32.1	1.9	32.0	12.9	30.0	2,000	693	0.66	15.1	0.908	100	15.1	0.908	2.1
30	Cancer of prostate. Metastatic to ribs, spine.	25.6	8.1	31.6		12.0	37.6	85.0	*2,945	1,015	0.90	46.2	1.898	81	37.1	1.525	3.6
31	Resected cancer of breast. Metastatic to ribs, spine.	34.0	10.6	31.2				97.5	*2,125	1,037	1.07	32.2	1.315	95	30.7	1.279	2.9
32	Anaplastic cancer of unknown primary site. Metastatic to liver, bone.	31.8	8.9	28.8		35.0	12.0	61.2	*2,793	1,191	0.58	45.7	1.599	93	42.5	1.487	3.6
33	Cancer of testes. Metastatic to brain, retroperitoneal nodes.	47.0	14.4	30.1				108.0	*2,292	1,742	1.33	30.9	0.739	87	27.8	0.665	1.6
34	Cancer of lung. Metastatic to bone.	39.0	11.9	30.5													
35	Cancer of prostate. Metastatic to bone (on estrogen Rx).	30.2	10.0	32.5	1.8												
36	Cancer of lung. No metastases.	40.0	14.0	34.6													
37	Resected cancer of stomach. Metastatic to bones.	21.0	6.3	31.0													
38	Resected cancer of sigmoid. Metastatic to liver, peritoneum.	30.0	10.0	33.0													

TABLE II

Correlation of the clinical status of the cancer with the severity of the anemia

Status of disease	Number of cases	Anemia present*	Anemia mild	Anemia moderate	Anemia severe
1) Localized	4	None	None	None	None
2) Metastases present	34	22	6	14	2
(a) Metastases to bone	18	17	6	9	2

* Anemia mild (10.5 to 12.0 gm. per cent).
moderate (7.0 to 10.4 gm. per cent).
severe (<7.0 gm. per cent).

nique of Ashby as modified by Ebert and Emerson (18) and Young, Platzer, and Rafferty (19) was employed. Net inagglutinable counts were expressed as per cent transfused cells remaining at time (t) using the net inagglutinable counts at 24 to 48 hours as the 100 per cent point. The mean life span of transfused red cells was roughly estimated by drawing a straight line through the main slope of the survival curve and noting where it intercepted the time axis.

(b) *Chromium red cell survival studies.* The survival of the patient's own red cells was determined by the Cr^{51} method of Ebaugh, Emerson, and Ross (20) in two patients. Both blood and plasma samples were secured and counted 24 and 48 hours after transfusion. Thereafter, counts on whole blood were performed at intervals of 2 to 3 days for the first 2 to 3 weeks and subsequently once every 1 to 2 weeks. Corrections for chromium elution were made as recommended by Ebaugh, Emerson, and Ross (20).

5) *Iron metabolism studies*

(a) *Serum iron determination.* The serum iron concentration was determined by the method of Kitzes, Elvehjem, and Schuette (21). Normal levels by this technique in our laboratory were 70 to 155 γ per cc.

(b) *Tissue iron demonstration.* Sections of liver, spleen and bone marrow were stained for iron by the Berlin blue stain for hemosiderin on patients expiring of their disease.

(c) *Radioiron turnover studies.* Fe^{59} secured from Abbott Laboratories, Oak Ridge, Tennessee, with specific activity of 2 to 5 μc. per μg. was converted from ferric chloride to ferric ammonium-citrate. Then, 0.1 to 0.3 cc. of this sterilized material containing about 2.0 to 7.0 μg. of iron with a radioactivity of 7 to 11 μc. was incubated at 37° for 30 minutes with 12 to 13 cc. of plasma obtained from a normal human subject. A 10-cc. aliquot of this plasma was injected intravenously from a calibrated syringe and needle. The disappearance of Fe^{59} from the plasma, the plasma iron turnover, the red cell utilization of Fe^{59}, the red cell iron turnover and the red cell iron renewal rate were determined by the method of Huff, Hennessy, Austin, Garcia, Roberts, and Lawrence (22). The plasma and red cell turnover was expressed as the $\frac{\text{mg. iron/hr.}}{\text{liter red cell mass}}$ and calculated from the following formula:

$$\text{Plasma iron turnover} = \frac{0.693 \times \text{serum iron conc. } (\mu\text{g. per cc.}) \times \text{plasma vol. (cc.)}}{T_{\frac{1}{2}} \text{ (in hours)} \times 1{,}000 \times 24 \text{ liter red cell mass}}$$

$$\text{Red cell iron turnover} = \frac{\text{red cell iron turnover mg. per hr.}}{\text{liter red cell mass}}$$

The control values on 8 normal subjects to be alluded to are from Pollycove, Apt, and Ross (23). The experimental plan in the performance of these normal studies is similar to that used in the present study.

6) *Distribution and movement of Fe^{59} in the body*

Following the intravenous injection of radioactive iron, the localization of radioactivity in various organs was measured. Measurements of radioactivity were made over the heart, liver, spleen, sacrum and thigh at 30 to 60 minutes, 2, 4, and 6 hours, and at daily intervals over a period of 1 to 2 weeks.

7) *Measurement of radioactivity*

(a) *Beta counting.* Radioactive phosphorus labelled erythrocytes were counted as solid samples using a thin window beta-sensitive Geiger-Müller tube. The counting error was less than 1 per cent.

(b) *Gamma counting.* Fe^{59} and Cr^{51} samples were counted in the liquid state using a well-type scintillation counter (sodium iodide, thallium activated). When red cell samples were counted, saponin was added to produce hemolysis. Samples usually counted 5 to 15 times background and sufficient total counts were taken to give a statistical counting error of 1 to 2 per cent. In the 3 or 4-hour plasma samples from the plasma iron turnover studies, the concentration of Fe^{59} was often so low that the statistical counting error was 5 to 10 per cent. External monitoring measurements were made with a one inch diameter, one inch deep thallium activated, sodium iodide scintillation crystal applied directly to the skin. Each site was counted for 1-minute intervals while the background was counted for 10 minutes. The background count ranged from 600 to 1,000 counts per minute and the body counts usually ranged from 6,000 to 18,000 counts per minute.

EXPERIMENTAL RESULTS

1. *Hematological*

Of the patients studied, 22 out of 38 had an anemia, with the severity of the anemia listed in Table II. In 19 of the 22, the anemia was normochromic with a mean corpuscular hemoglobin concentration (MCHC) of 30 per cent or above (Table I). The remaining 3 patients had slightly reduced MCHC's ranging from 28.8 to 29.9 per cent. Twelve of the 16 patients without anemia had metastases and, despite extensive bone metastases, one of the 16 had normal hemoglobin values. The reticulocyte count was slightly elevated (3 to 5 per cent) in 4 patients. The Coombs test was negative and the serum bilirubin was within normal limits in all patients so studied.

2. *Radioiron studies*

(a) *Plasma disappearance half-time of Fe^{59} ($T\frac{1}{2}$).* The plasma disappearance half-time of Fe^{59} ($T\frac{1}{2}$) ranged from 1.18 to 2.18 hours in normal subjects. In 27 of 37 studies on 33 cancer patients the $T\frac{1}{2}$ was more rapid than normal (Table I). The $T\frac{1}{2}$ appeared to correlate fairly well with serum iron levels, the coefficient of correlation being 0.690 (Figure 1). Thus short $T\frac{1}{2}$'s were present when serum iron levels were depressed, and long $T\frac{1}{2}$'s when serum iron levels were elevated. The relationship between $T\frac{1}{2}$ and serum iron concentration was further noted when repeat radioiron studies were performed on the same patient after progression of the disease had occurred. Patient No. 12 was first studied when her disease was relatively quiescent and when the $T\frac{1}{2}$ and the serum iron were both normal (Table I). Seventy-three days later, when her disease had become widely disseminated, a depressed serum iron level and a short $T\frac{1}{2}$ was present. A similar finding was present in patient No. 29, who, with slow progression of her disease, had progressive lowering of serum iron levels concomitant with shorter $T\frac{1}{2}$'s (Table I). Short $T\frac{1}{2}$'s were not a good index of increased erythropoiesis. Thus in 17 of 37 studies, with slow $T\frac{1}{2}$'s the daily rates of red cell production were either normal or actually decreased as measured by the red cell iron turnover (Table I). In only 9 out of 37 studies were the $T\frac{1}{2}$'s within normal limits. Four patients in this group had quiescent disease, and anemia was present in only 2 such patients. Patient No. 17, with an elevated serum iron and decreased red cell production, had a longer than normal $T\frac{1}{2}$.

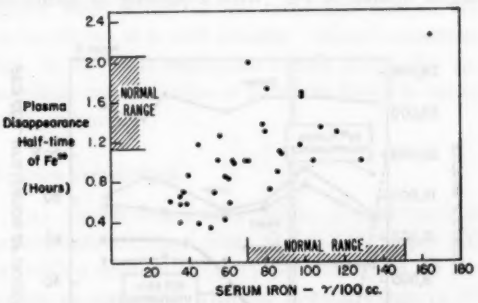

FIG. 1. CORRELATION OF THE Fe^{59} PLASMA DISAPPEARANCE HALF-TIME WITH THE SERUM IRON CONCENTRATION IN 33 PATIENTS WITH MALIGNANT NEOPLASMS

Each point in the above chart represents the serum iron concentration and the plasma disappearance half-time of a patient with malignant disease. The coefficient of correlation between the above two measurements was 0.690.

(b) *Plasma iron turnover.* In 21 of 37 studies the plasma iron turnover (the amount of iron leaving the plasma per unit time) was within the normal range of 0.569 to 1.149 mg. iron per hour per liter red cells (Figure 2). In 11 patients the plasma iron turnover rate was increased over the normal, and in patients No. 1 and 27, the increase was 2 to 4 times normal. In only 2 of 37 studies was the plasma iron turnover decreased.

(c) *The red cell utilization of injected Fe^{59}.* The incorporation of the injected Fe^{59} into newly formed red cells was normal (80 to 100 per cent) in 29 of 36 studies on 32 patients (Table I). Despite this normal red cell utilization of Fe^{59} in patients No. 16, 20 and 21b, the daily rate of red cell production was decreased when the red cell iron turnover was calculated. In only 7 of 36 studies was the red cell utilization of Fe^{59} decreased. Only 5 per cent red cell utilization occurred in patient No. 17, who had the long $T\frac{1}{2}$.

(d) *The red cell iron turnover.* In 20 of 36 studies the red cell iron turnover (the amount of iron entering into newly formed red cells per unit time) was within the normal range of 0.569 to 1.033 mg. iron per hour per liter red cells (Figure 2). In 11 studies the red cell iron turnover was increased. A 2 to 4 fold increase over normal oc-

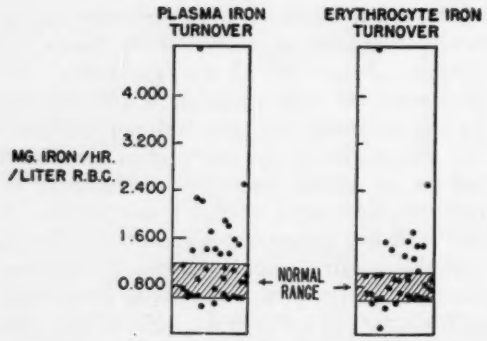

FIG. 2. THE RATE OF PLASMA IRON TURNOVER AND THE RATE OF RED BLOOD CELL IRON TURNOVER IN 33 PATIENTS WITH NEOPLASTIC DISEASE

curred in patients No. 1 and 27. A definite increase in red cell iron turnover was also noted in patients No. 9 and 25 despite the presence of normal hemoglobin levels (Table I). A decrease in red cell iron turnover was noted in 5 patients ranging from 0.114 to 0.534 mg. iron per hour per liter red cells. Anemia was present in only 2 of these patients. A profound decrease in red cell iron turnover was noted in patient No. 17 alluded to previously, in whom subsequent autopsy showed complete bone marrow replacement with lymphosarcoma.

(e) *Red cell renewal rate.* The red cell renewal rate (the fraction of red cells renewed daily) was increased over the normal rate of 1.1 to 1.8 per cent in 20 studies on 18 patients (Table I). A very marked increase in red cell renewal rate, *i.e.*, 4 to 8 times normal, was noted in patients No. 1 and 27 associated with a 2 to 4 fold increase in red cell production. Patients No. 9 and 25, with normal hemoglobin values and increased red cell iron turnover values, demonstrated red cell renewal rates that were almost twice normal. In all twenty studies with increased red cell renewal rates metastases were present, whereas metastases were discovered in only nine of the sixteen studies with normal renewal rates. Anemia was present in seventeen of the twenty cases with increased red cell renewal rates, whereas it was observed in only three of the sixteen cases with normal renewal rates.

3. *External monitoring*

The localization of iron in the body, and changes in this localization with the passage of time, were studied by external monitoring in 8 patients. An early rise in sacral activity followed by a gradual decline (Figure 3) occurred in 5 patients (No. 11, 12, 15, 16 and 20). This is thought to be representative of functioning erythropoietic bone marrow and is similar to the pattern found in normal subjects. Three other patients (No. 5, 17 and 29) demonstrated abnormalities in the distribution of Fe^{59}. Patient No. 5, whose hemolytic index was increased, showed an early and sustained concentration of Fe^{59} over the spleen without a sacral concentration of Fe^{59} (Figure 4). Patient No. 17, whose bone marrow was replaced by lymphosarcoma, also demonstrated a failure of sacral uptake of Fe^{59} with a pooling of radioiron

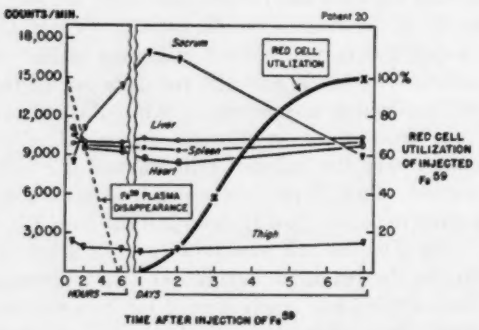

FIG. 3. NORMAL PATTERN OF Fe^{59} DISTRIBUTION IN A PATIENT WITH FIBROMYXOSARCOMA

Note the early rise and subsequent decline in sacral radioactivity.

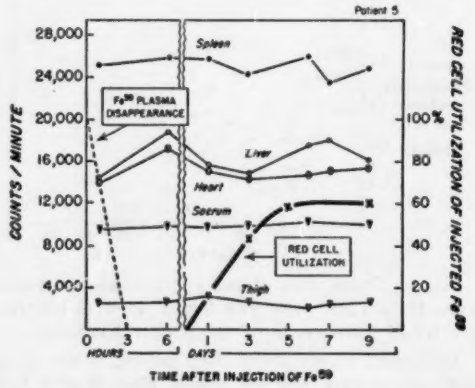

FIG. 4. SPLENIC CONCENTRATION OF Fe^{59} IN A PATIENT WITH AN ELEVATED HEMOLYTIC INDEX

Note also the absence of sacral uptake of Fe^{59}.

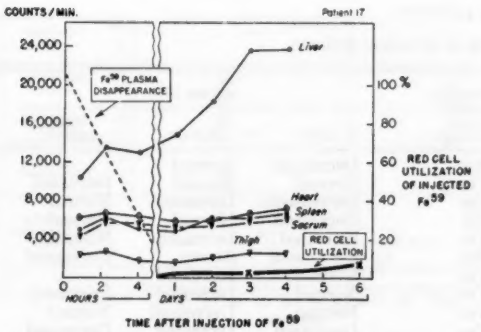

FIG. 5. HEPATIC POOLING OF Fe^{59} IN A PATIENT WITH BONE MARROW REPLACEMENT BY LYMPHOSARCOMA
Note also the absence of sacral concentration of Fe^{59}.

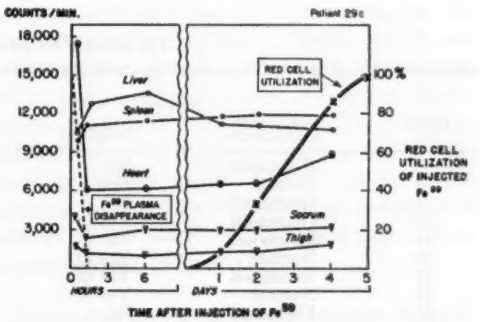

FIG. 6. ABSENCE OF SACRAL CONCENTRATIONS OF Fe^{59} IN A PATIENT WITH WIDESPREAD LUMBOSACRAL BONE METASTASES

in the liver (Figure 5). Lastly, patient No. 29 with wide-spread lumbosacral bone metastases, demonstrated a failure of sacral uptake unassociated with any increase in liver or spleen Fe^{59} uptake (Figure 6). Despite the absence of sacral concentration of Fe^{59}, this patient had normal red cell utilization of Fe^{59}.

In patients No. 20 with a fibromyxosarcoma in the right scapular region, and No. 15 with cancer of the left breast, external monitoring revealed no appreciable concentration of the injected Fe^{59} in the tumor areas. In patient No. 10 with bronchogenic carcinoma, and patient No. 11 with adenocarcinoma of the bronchus, Fe^{59} was injected 24 hours before surgery. From the resected tumor, 4 to 8-gm. aliquots of the entire tumor were placed in test tubes and assayed for radioactivity in a well counter. Blood concentration of Fe^{59} was negligible at this time. No appreciable concentration of Fe^{59} was found in either of these tumors.

4. *Iron metabolism*

(a) *Serum iron concentration.* The serum iron concentration ranged from 30.0 to 165.0 γ per 100 cc. with a median of 65.0 γ per 100 cc. in 33 patients, as compared with the normal range of 70 to 155 γ per 100 cc. Depressed serum iron levels were found in 17 patients. The incidence and the degree of depression could not be correlated with the presence or extent of metastatic tumor. Thus, 11 of the 16 patients with normal serum iron levels had widespread disease, as compared with 12 of 17 patients with widespread disease who had depressed serum iron values. However, when measurements were repeated during the course of tumor spread, a progressive fall in serum iron concentration could usually be demonstrated. This phenomenon is well illustrated in case No. 12, who, when first studied, was asymptomatic and had a normal serum iron. Seventy-three days later, following widespread dissemination of the disease process, the patient's serum iron had decreased to 34.6 γ per 100 cc. (Figure 7). Six other patients demonstrated similar lowering of serum iron levels with progression of their disease. Contrariwise, with improvement in the clinical state of the patient, serum iron levels tended to rise towards normal (Figure 7). Thus, in patient No. 10 with bronchogenic carcinoma without metastases, the serum iron concentration was 35.0 γ per 100 cc. at the time of the first study. Eight months later, following complete surgical removal of the carcinoma, the serum iron had risen to a low normal level of 89.7 γ per 100 cc.

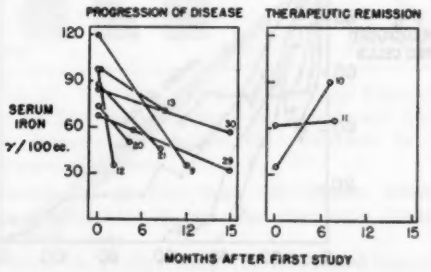

FIG. 7. CHANGES IN SERUM IRON LEVELS ASSOCIATED WITH CHANGES IN THE CLINICAL STATUS OF MALIGNANT NEOPLASMS IN NINE PATIENTS

TABLE III

The status of the iron stores in 10 cancer patients

Patient No.	Serum iron concentration	Transfused	Evidence of increased red cell destruction	Storage iron		
				Liver	Spleen	Bone marrow
3	Decreased	No	No	Decreased	Normal	
4	Normal	No	Yes	Normal	Normal	Increased
7	Decreased	No	Yes	Decreased	Increased	Normal
20	Decreased	Yes (4)*	No	Decreased	Normal	Normal
23	Decreased	No	Yes	Increased	Increased	Normal
26	Normal	Yes (2)	Yes	Decreased	Absent	Decreased
27	Decreased	Yes (2)	Yes	Normal	Increased	
31	Normal	Yes (2)	Yes	Normal	Increased	Increased
32	Decreased	No	Yes	Normal	Decreased	Normal
34		Yes (2)	Yes	Decreased	Increased	Decreased

* Number of pints of transfused blood.

Anemia and hypoferremia were associated findings in 10 of 33 patients (Table I). Anemia in the presence of a normal serum iron concentration was found in 7 patients, while hypoferremia without anemia occurred in 6 other patients. Ten patients displayed neither anemia nor hypoferremia.

5. *Determination of storage iron*

A semi-quantitative estimate of storage iron was accomplished in 10 cases by examining postmortem sections of liver, spleen and bone marrow stained for hemosiderin with Berlin blue. In 7 of 10 patients the iron stores appeared to be normal, while in two others slightly increased amounts were found (Table III). A decrease in the quantity of storage iron was found in patient No. 22. Despite the presence of normal amounts of storage iron, the serum iron concentration was decreased in 6 patients. Two of the 3 patients, who had decreased mean corpuscular hemoglobin concentrations (No. 23 and 32), had normal or increased amounts of storage iron. The one patient with the decreased amount of storage iron had a low normal serum iron level.

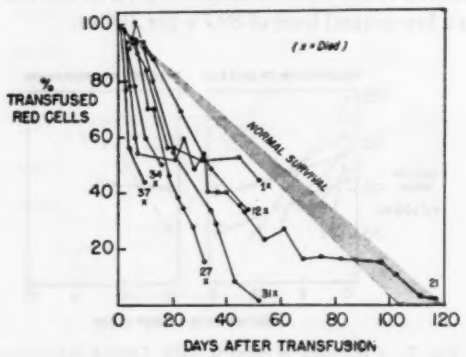

FIG. 8. INCREASED DESTRUCTION OF NORMAL TRANSFUSED RED CELLS IN SEVEN CANCER PATIENTS

6. *Fecal urobilinogen studies*

The hemolytic index was elevated in 6 of 13 patients (Table I). In five of these 6 patients, additional evidence for increased red cell destruction was secured from measurements of red cell survival and Fe^{59} studies.

7. *Red cell survival studies*

Increased red cell destruction was found in 5 patients, the life span of transfused erythrocytes ranging from 25 to 80 days, in contrast to the

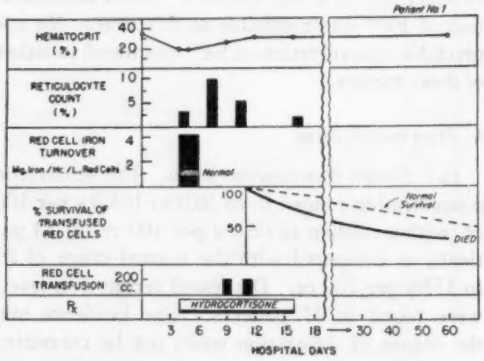

FIG. 9. HEMATOLOGICAL, RADIOIRON, AND RED CELL SURVIVAL STUDIES ON A PATIENT WITH DISSEMINATED CARCINOMA OF THE PROSTATE

See text for explanation.

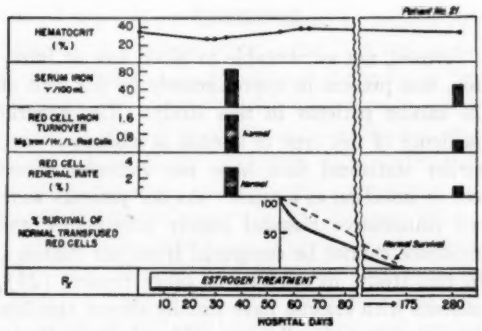

FIG. 10. HEMATOLOGICAL, RADIOIRON, AND RED CELL SURVIVAL STUDIES ON A PATIENT WITH WIDESPREAD CARCINOMA

See text for explanation.

normal 120-day survival period (Figure 8). Patients No. 1 and 21 destroyed donor cells rapidly during the initial phase of the study, the rate of hemolysis subsequently returning to normal. As shown in Figure 9, during the first 18 days following transfusion, patient No. 1 eliminated the donor cells at a rate of 2.8 per cent per day, the daily loss thereafter being reduced to 0.85 per cent. Patient No. 21 showed a daily loss of donor cells of 5.6 per cent per day for the first 10 days, and a normal red cell survival subsequently (Figure 10). Red cells survived normally in 5 patients (No. 24, 29, 35, 36, and 38), 4 of whom had metastases and anemia. Patient No. 38, in the face of severe anemia and disseminated cancer,

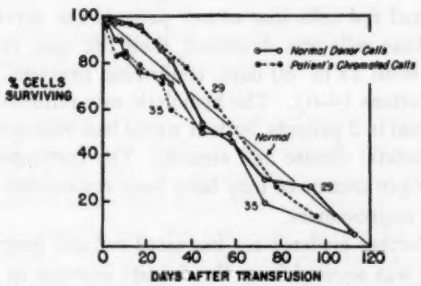

FIG. 11. THE SURVIVAL OF NORMAL DONOR RED CELLS AND THE RED CELLS OF TWO PATIENTS WITH DISSEMINATED CARCINOMA

The above data indicate that the survival of normal transfused red cells measured by the red cell differential agglutination technique, and the patient's red cells measured by the Cr^{51} technique, were both normal.

exhibited a normal red cell survival during the terminal month of his illness. Patient No. 29 was studied on two occasions, 11 months apart, during which time hemoglobin levels were stable despite the progression of the carcinoma. Both studies were normal. The survival of normal donor cells, based on Ashby counts and the patient's own erythrocytes labelled with Cr^{51}, were measured in patients No. 29 and 35. The results were normal in each instance (Figure 11).

8. *Serial observation during the advance of cancer*

(a) Patient No. 12 had had a radical mastectomy for cancer of the breast three years prior to this study. A solitary metastasis to the left orbit, first discovered eight months previously, was the only evidence of dissemination of the cancer. At the time of the first Fe^{59} study, the patient was asymptomatic, with normal serum iron and hemoglobin levels, normal $T\frac{1}{2}$ of Fe^{59} and normal rates of red cell production and destruction (Figure 12). Studies were repeated seventy-three days later when there was obvious dissemination of the cancer to lungs, liver and femur. Anemia had developed, the concentration of serum iron had de-

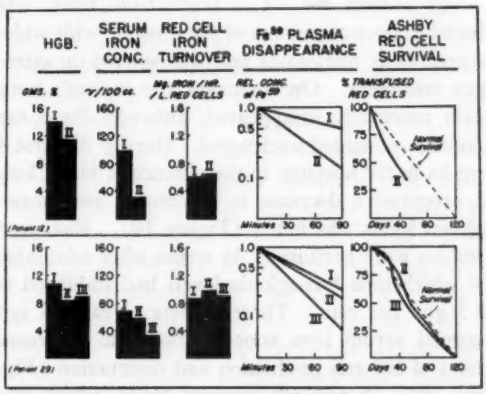

FIG. 12. THE EFFECT OF RAPID AND SLOW DISSEMINATION OF METASTATIC CANCER ON HEMOGLOBIN LEVELS, IRON METABOLISM AND RED CELL SURVIVAL IN TWO DIFFERENT PATIENTS

Patient No. 12 First study (I) Disease quiescent; Second Study (II) 73 days later after rapid dissemination of carcinoma.

Patient No. 29 First study (I) Widespread bone metastases present; Subsequent study (II) 4 months later, no clinical change; *Final study* (III) 11 months later after the appearance of new bone metastases.

clined and the $T\frac{1}{2}$ of Fe^{59} had become rapid. The red cell iron turnover was discovered to be increased, but the red cell renewal rate had not changed. A survival study demonstrated increased red cell destruction, the estimated life span of normal donor cells averaging between 75 and 80 days (Figure 12). The patient died 48 days after the transfusion study had started.

(b) Patient No. 29, had had a radical mastectomy in 1948 for a cancer of the breast. The patient was studied three years later when metastases were widespread. The first study revealed anemia, a slight reduction of serum iron, a moderately short $T\frac{1}{2}$ of Fe^{59}, normal red cell production and a questionable increase in red cell destruction (Figure 12). Four months later, her clinical status having remained stationary, the patient was re-studied. A moderate decline in hemoglobin and serum iron levels and a reduction of $T\frac{1}{2}$ of Fe^{59} were discovered. A survival study demonstrated normal life span of the transfused red cells. Eleven months later, new skin and bone metastases were present. The serum iron showed further depression, and shortening of the $T\frac{1}{2}$ was more pronounced, whereas the survival of donor and patient's cells remained normal.

(c) Patient No. 21, a 62-year-old male, was found to have carcinoma of the prostate with widespread bone metastases and was started on estrogen treatment. On treatment the patient's bone pain practically disappeared, although the x-ray findings remained unchanged. During the first 4 weeks in the hospital, in the absence of blood loss, a progressive decrease in hematocrit and hemoglobin levels was noted (Figure 10). Radioiron studies were performed $4\frac{1}{2}$ weeks after admission at which time hemoglobin levels had stabilized at 8.5 gm. per cent. These studies revealed a low normal serum iron concentration and increased rates of red cell production and destruction (Figure 10). A red cell survival study, which was begun at a time when the hemoglobin and hematocrit levels were slowly rising, revealed increased red cell destruction during the first 10 days, but thereafter the remaining transfused cells survived normally. Eight months later, the disease having been relatively static, the serum iron was depressed and the $T\frac{1}{2}$ of Fe^{59} shorter. Red cell production was now slightly reduced but red cell destruction, previously rapid, was normal.

DISCUSSION

Anemia, not attributable to blood loss or infection, was present in approximately 60 per cent of the cancer patients in this study. The general incidence of this type of anemia is unknown since earlier statistical data have not excluded blood loss or infection as a cause. As our patients were not consecutive hospital cancer admissions, the incidence cannot be computed from our findings. In this study, in contrast to other reports (24), patients with anemia have usually shown absolute decreases in red cell mass. Morphologic observations on the bone marrow usually revealed normal to increased cellularity, despite the presence of marrow metastases and anemia. This is similar to the observations of other investigators who have found no correlation between the presence of anemia, the cellularity of the bone marrow, or the extent of bone marrow metastases (1–3).

Evidence pointing to increased red cell destruction as one of the mechanisms responsible for this anemia has been secured from fecal urobilinogen, red cell survival and Fe^{59} studies. In 2 patients with evidence of increased hemolysis from radioiron data, the hemolytic index calculated from the fecal urobilinogen excretion was normal. The relative insensitivity of fecal urobilinogen analysis in demonstrating minor increases in daily red cell destruction as measured by the red cell survival techniques has also been noted by Ross, Crockett, and Emerson (7) in their studies in leukemia patients.

In 5 of 13 studies involving the transfusion of normal red cells into cancer patients the survival of these cells was shortened, their life span ranging from 25 to 80 days, confirming previous observations (4–6). The hemolytic rate returned to normal in 2 patients, both of whom had widespread metastatic disease and anemia. The cortisone or estrogen treatment may have been responsible for this improvement.

Further evidence for increased red cell destruction was secured from the definite increase in red cell renewal rates in two-thirds of the patients studied. Patients with very low serum iron levels and rapid $T\frac{1}{2}$'s of Fe^{59}, who show slight increases in red cell renewal rates, do not necessarily have increased rates of red cell destruction as exemplified by patient No. 29. The apparent increase in

red cell renewal rate in such cases is probably explained by a technical error in the Fe^{59} technique. Possible sources of error include difficulty in the following: (a) measuring the $T\frac{1}{2}$ accurately when it is brief; (b) variation in results obtained by the Kitzes, Elvehjem, and Schuette (21) method of determining serum iron, the error approaching 10 per cent for levels between 30 and 70 γ per 100 cc. of serum, levels which are typical of cases with rapid $T\frac{1}{2}$'s. The $T\frac{1}{2}$ and the serum iron concentration are two of the chief parameters in the calculation of red cell renewal rates, and errors in the estimation of either may be responsible for the discrepant finding of a rapid red cell renewal rate in patients without other evidence of increased red cell destruction. Even if this small group of patients were eliminated from consideration, more than half of the patients studied demonstrated evidence of increased red cell destruction.

The mechanism of increased hemolysis in cancer patients is unknown. Coombs tests failed to demonstrate any antibody mechanism which might have played a role in the increased red cell destruction, our experience in this regard paralleling that of Hyman (6). Weil (25) and Gross (26, 27) reported that extracts of tumor tissue hemolyzed red cells *in vitro*. Whether or not an analogous situation exists *in vivo* causing injury to circulating red cells and chronic hemolytic anemia is another possibility to be explored. Large doses of x-ray and radium used in the treatment of cancer of the cervix have been reported to increase the rate of red cell destruction (5). In as much as some of our patients with increased red cell destruction had received irradiation therapy prior to study, a causal relationship of this type cannot be excluded.

Hypoferremia was a frequent but not invariable finding in cancer patients, as others have reported (8–10, 28), the depression of plasma iron concentration tending to parallel in degree the extent of growth and the metastatic spread of the neoplasm. The fact that the serum iron concentration rose in patient No. 10 following surgical removal of tumor tissue is evidence that the tumor itself was in some way responsible for the hypoferremia.

The finding of normal or increased amounts of hemosiderin iron in sections of liver, spleen, and bone marrow tends to exclude iron deficiency as the cause of hypoferremia and anemia in our patients. Sandberg, Gross and Holly (29, 30) reported normal to increased amounts of iron in the liver and spleen of patients dying with disseminated carcinoma and anemia, the amount often appearing to be excessive in proportion to the degree of anemia. Our findings partially confirm these observations, although the quantity of iron found in our cases was less. Since the organs analyzed by Sandberg, Gross and Holly contained indeterminate amounts of hemoglobin iron, their large values might be explained in part on that basis.

The depression of serum iron levels in the presence of normal or slightly increased storage iron in cancer has its analogy in acute and chronic infections (31, 32) but the mechanism responsible for the phenomenon is not understood. Depletion of serum iron as a result of its extraction by neoplasms with an increased avidity for that element is unlikely in view of the fact that accumulation of Fe^{59} in tumor tissue has not been demonstrable in any of the cases we have investigated from this standpoint (Cases No. 10, 11, 15 and 20). Moreover, chemical analyses of various malignant tumors described in the literature (30, 33, 34) have demonstrated that the concentration of iron in such tissues is low. There is evidence to incriminate the reticulo-endothelial system in the genesis of the hypoferremia found in experimentally produced acute inflammation in the dog (35, 36). Whether a similar mechanism is operative in the chronic cancer patient is uncertain.

The $T\frac{1}{2}$ of injected Fe^{59} was abnormally short in two-thirds of the patients studied. A fair correlation existed between serum iron levels and the $T\frac{1}{2}$, with a prolonged $T\frac{1}{2}$ associated with an increased serum iron, and a short $T\frac{1}{2}$ with a decreased serum iron. That this is not uniform is seen in conditions like pernicious anemia, hemolytic anemias, and infectious hepatitis (37, 38) where normal to elevated serum iron values may coexist with short $T\frac{1}{2}$'s. Contrary to the observations of Wasserman, Rashkoff, Leavitt, Mayer, and Port (37), short $T\frac{1}{2}$'s do not always signify increased rates of erythropoiesis. It would appear that the disappearance rate of injected Fe^{59} is predominantly affected by two factors: the level of serum iron, and the rate of erythropoiesis. A major change in either or both factors alters the

$T\frac{1}{2}$. Thus, depression of the serum iron from either iron deficiency, cancer, or infection is associated with a short $T\frac{1}{2}$ (*37*), whereas with elevation of the serum iron, as in hemochromatosis, a long $T\frac{1}{2}$ is found (39). When erythropoiesis is increased, as in polycythemia vera or hemolytic anemia, a short $T\frac{1}{2}$ is to be expected (*23, 37*); conversely, in the hypoplastic anemias the $T\frac{1}{2}$ is prolonged (*23, 37*).

External monitoring revealed normal patterns of Fe^{59} distribution in 5 patients, 2 of whom were anemic. Three other anemic patients exhibited abnormal localization sites of the injected Fe^{59}. One of these, with extensive metastases involving the lumbosacral spine, failed to concentrate the Fe^{59} in the sacral marrow despite normal red cell utilization of the radioiron. Bone marrow sites uninvolved with tumor must have accounted for the normal utilization of Fe^{59}. Thus, impaired utilization of Fe^{59} by the total bone marrow cannot be assumed from the absence of sacral concentration of Fe^{59} in patients with metastatic neoplasms. Another patient with signs of increased red cell destruction promptly proceeded to concentrate Fe^{59} in the spleen to a striking degree, the sacral concentration characteristic of normal subjects failing to appear. The spleen in this patient may have served as an erythroblastic organ, which could explain the early Fe^{59} uptake. The high spleen concentration of Fe^{59} for the first nine days may reflect erythroclastic activity, *i.e.*, destruction of newly formed red cells labelled with Fe^{59}. Combination erythroblastic-erythroclastic spleens have been described by Elmlinger, Huff, Tobias, and Lawrence (40) in myelofibrosis and cirrhosis with superimposed hemolytic anemia. In a third patient with diffuse marrow replacement by lymphosarcoma and marked depression of erythropoiesis the sacrum bone marrow failed to concentrate Fe^{59}, whereas a progressive and sustained pooling of the isotope occurred in the liver. Presumably this represents the storage of Fe^{59} that is not to be used for hemoglobin production by the tumor-ridden marrow and resembles the pattern described by Elmlinger, Huff, Tobias, and Lawrence (40) in patients with refractory anemia and decreased erythropoiesis.

It would appear that anemia in cancer may be caused by different pathophysiological mechanisms. For example, increased red cell destruction, associated with normal or increased rates of red cell production, is commonly found in these cases. The concept of bone marrow depression due to "replacement" by cancer cells or "toxic" depression by a tumor metabolite is not substantiated by this study. Although red cell production may be increased 2 to 4 fold, it may not keep pace with the increased red cell destruction, the hemoglobin concentration declining to the level at which production and destruction rates are matched. A characteristic abnormality of the patient with cancer is failure to compensate fully for an increased rate of red cell destruction, whether the latter is mild or severe. Thus, the 6 to 8 fold increase in erythropoietic activity displayed by a normal bone marrow after acute hemorrhage, or by the bone marrow of a patient with hereditary spherocytosis (41) in response to anemia, is not seen in cancer. In spite of morphological and physiological evidence of hyperactive erythropoietic activity, the marrow of the cancer patient actually shows "functional inadequacy" in the face of demand. That this apparent failure may not be absolute is suggested by the reticulocyte response and the rise in hemoglobin in some anemic cancer patients treated with cobalt (1). Moreover, a few of these patients are capable of compensating for increased hemolysis without anemia developing. This state of hemolysis in cancer has also been described by Sheets, Hamilton, DeGowin, and Janney (5).

As had been assumed in the past the anemia associated with cancer can result primarily from a decrease in red cell production. This situation is most apt to arise in patients with widespread metastases but can occur without gross evidence of marrow involvement. However, the concept of bone marrow replacement as a common cause of anemia is untenable even when decreased red cell production can be demonstrated. It is possible that inadequacy of marrow activity in cancer, whether relative or absolute, merely reflects an alteration in the homeostatic mechanism which determines the level of hemoglobin concentration or red cell mass in the tumor host.

SUMMARY

1. Evidence of increased red cell destruction based on measurements of fecal urobilinogen ex-

cretion was found in 6 of 13 patients with malignant neoplasms. Increased red cell destruction was also found in 7 of 12 patients by red cell survival studies.

2. The rate of red cell production, as measured by radioiron and red cell survival studies, was normal or increased in 29 patients in this series, but usually failed to compensate for the increased hemolysis with anemia resulting. Thus, a "functional inadequacy" of erythropoiesis in the face of a red cell deficit may be considered the fundamental mechanism of anemia in neoplastic disease.

3. Anemia was solely attributable to deficient red cell production in 9 patients, 4 of whom were totally or nearly free of marrow metastases.

4. Hypoferremia associated with normal quantities of storage iron was a common finding in patients with cancer, the concentration of serum iron becoming progressively lower with dissemination of the tumor. Hypoferremia was usually, but not invariably, accompanied by anemia.

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THYROXINE-BINDING BY SERA OF PREGNANT WOMEN, NEWBORN INFANTS, AND WOMEN WITH SPONTANEOUS ABORTION [1]

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Among the many physiological alterations which may occur during pregnancy are thyromegaly (1), augmented thyroidal avidity for iodine (?, 3), and increase in the concentration of circulating thyroid hormone (4, 5). This triad of anatomical and functional alterations is ordinarily considered to be diagnostic of thyrotoxicosis. Paradoxically, however, these findings are neither accompanied by symptomatic stigmata of hyperthyroidism, nor are they associated, during the first half of pregnancy, with increase in the basal metabolic rate. Although the basal metabolic rate increases during the latter half of pregnancy, this change has been ascribed by some to fetal needs, and not to alterations in maternal energy requirements (6). The origins and consequences of this unique functional dissociation are unknown.

Recently there has been considerable interest in the physiological role and physicochemical properties of the specific thyroxine-binding protein or proteins of plasma (TBP). Although the physiological role of TBP has not been elucidated, demonstration of this alpha globulin moiety in 1952 provided another parameter of thyroidal economy which is susceptible to measurement (7–11). Even earlier it had been suggested that the increased SPI of pregnancy might result from an alteration in the manner in which thyroxine in plasma is protein-bound (12, 13). Therefore, it appeared possible that study of the thyroxine-binding capacity of TBP in serum might provide a clue to the origin of the peculiar alterations in the metabolism of iodine which occur during pregnancy. This possibility seemed particularly attractive in view of repeatedly demonstrated alterations, during pregnancy, in the electrophoretic composition of the proteins of plasma (14–16).

For these reasons the present study of the interaction of thyroxine with the proteins of sera obtained from pregnant women was undertaken. A marked increase has been found in either the concentration of TBP, or in its avidity for thyroxine. It is suggested that this increase may be directly related to the alterations in the metabolism of iodine which occur in the pregnant woman. A brief preliminary report of this finding has been published elsewhere (17).

MATERIALS AND METHODS

Thirty ml. of venous blood were obtained without stasis from 27 non-pregnant women of childbearing age, without regard to the phase of the menstrual cycle, from 22 pregnant women divided among the three trimesters of uncomplicated pregnancy, and from 6 pregnant women with a diagnosis of threatened or inevitable abortion. In an additional 8 instances, maternal and umbilical cord bloods were obtained at the time of normal, full-term delivery. Other samples studied included venous blood drawn serially from 3 patients at delivery and during the first, third, and fifth post-partum weeks, and blood from 2 pregnant women at the estimated twenty-first postovulatory day. Serum was obtained following clot retraction. Aliquots of serum were immediately analyzed for albumin and for total protein. The remaining serum was frozen until used for studies of thyroxine-binding.

Concentrations of albumin in the sera were determined, in duplicate, by the colorimetric, dye-adsorption method of Rutstein, Ingenito, and Reynolds (18). Standard curves of optical density were obtained, using a Coleman Junior spectrophotometer. The following preparations of albumin were intercompared in order to obtain albu-

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min reference standards: Fraction V of Cohn method 6 (19), mercaptalbumin (20),[3] and two lots of 25 per cent ARC human serum albumin. Standards containing mercaptalbumin and Fraction V were prepared gravimetrically; standards of ARC human albumin were prepared by dilution. Close agreement was noted between colorimetrically determined values and the nitrogen content of the mercaptalbumin standard, as determined by Kjeldahl analysis. The reliability of using 25 per cent ARC human serum albumin as a standard for the method was thereby verified. A new standard curve was obtained for each set of determinations.

Concentrations of total protein were determined, in duplicate, by the biuret method (21), standardized by Kjeldahl determinations of nitrogen in the reference solutions of human albumin.

Analyses for serum precipitable iodine (SPI) were performed by the alkaline ash method of Barker, Humphrey, and Soley (22, 23).[4]

In order to assess the proportionate distribution of thyroxine between TBP and albumin at varying concentrations of thyroxine, sera to be compared in a single experiment were treated concurrently in the following manner. Into the first of 4 13 × 100 mm. test tubes were added 5.0 ml. of serum and 0.100 ml. of an approximately 5.0 μg. per ml. solution of newly received, chromatographically pure radiothyroxine.[5] Two ml. of this mixture was transferred to the fourth tube. Into the fourth tube was then added 0.100 ml. of a 3.0 ± 0.2 mg. per cent solution of stable thyroxine,[6] made up in 1.0 per cent human albumin. Solutions of stable thyroxine were prepared in 1.0 per cent albumin in order to minimize adsorption of thyroxine to glassware. In order to compensate for the albumin added to the fourth tube, 0.150 ml. of a 1.0 per cent solution of human albumin containing no thyroxine was added to the 3 ml. of serum remaining in the first tube. At this point, serum in the fourth tube contained approximately 150 μg. per cent of added stable thyroxine; serum in the first tube contained none. Serum in both the tubes contained approximately 50 mg. per cent of added human albumin. One-ml. aliquots of serum from the first and fourth tubes were then mixed in the third tube. Finally, 1.0-ml. aliquots of serum from the first and third tubes were mixed in the second tube. Thus, concentrations of added stable thyroxine of approximately 37.5 and 75.0 μg. per cent were achieved in the second and third tubes, respectively. Approximately 10 μg. per cent of additional thyroxine were contributed to the serum in each tube by the radiothyroxine added.

[3] Mercaptalbumin dialyzed against cysteine was kindly supplied by Dr. Margaret J. Hunter, Department of Biophysical Chemistry, Harvard Medical School.

[4] Performed by Bio-Science Laboratories, 2231 S. Carmelina Avenue, Los Angeles, California.

[5] Obtained from Abbott Laboratories, Oak Ridge, Tennessee.

[6] Kindly supplied by Dr. A. E. Hemming, Smith, Kline & French Laboratories, Philadelphia, Pennsylvania.

In most experiments, the concentrations of SPI achieved were chemically determined in two sets of sera. SPI values, obtained by dilution of enriched sera were not only higher than values predicted on the basis of gravimetric addition, but also were not consistent from experiment to experiment. However, the distribution of thyroxine between albumin and TBP in the same control sera showed only occasional variation when electrophoresed in different experiments. The latter finding suggested that concentrations of thyroxine achieved in the various experiments were, in fact, usually quite similar. It was thus decided to report, in the accompanying tables and figures, concentrations of added thyroxine based on gravimetric addition. However, in order to eliminate the effects of variations in preparative techniques as well as in the adsorption of thyroxine to glassware (24), each experiment was designed so that the partition of thyroxine in experimental sera could be directly compared with that observed in concurrently assayed controls.

Additional experiments were performed in which each sample of serum from the pregnant subjects was gravimetrically enriched with mercaptalbumin in amounts sufficient to achieve albumin concentrations equal to the highest concentrations among control sera. The partition of thyroxine was then assessed by the techniques described below. The binding affinity for thyroxine of the mercaptalbumin added was assayed by electrophoresis of this protein in solutions containing the four concentrations of thyroxine used in studying thyroxine-binding in serum.

Since all of the above experiments entailed the addition of sufficient radiothyroxine to increase the concentration of endogenous thyroid hormone by at least 100 per cent, further studies were performed with truly tracer amounts of the radioactive material. Sera from pregnant females and non-pregnant controls were diluted tenfold with saline. To 0.9-ml. aliquots of the diluted sera were added 0.1 ml. of a 10 gm. per cent albumin solution which contained a small amount of endogenous protein-bound iodine as well as 0.14 μg. per ml. and 4.3 μc. per ml. of added I^{131}-thyroxine. The resultant mixtures, in which it was hoped to approximate the concentrations of total protein, albumin, and PBI of the extracellular fluid, were then electrophoresed on paper.

Using apparatus and techniques described elsewhere (24), 32 individual electrophoretograms (samples from 8 patients) were obtained in duplicate daily on consecutive days until all samples had been electrophoresed. Earlier, it had been shown that no difference in the partition of thyroxine resulted when aliquots of the same sample of thyroxine-enriched serum were electrophoresed at intervals of 4 days. One filter paper sheet of a duplicate pair was subdivided into its 8 component electrophoretograms. These individual electrophoretograms were then scanned for radioactivity in an automatic strip scanner utilizing a gas-flow counter. The other sheet was radio-autographed on no-screen x-ray film and subsequently stained for proteins by the method of Durrum (25). In certain instances, the components of serum in

TABLE I

Distribution of thyroxine between TBP and albumin during filter-paper electrophoresis of sera from pregnant women

Subject	Thyroxine added (μg./100 ml.)* 10 TBP†	10 A‡	48 TBP	48 A	85 TBP	85 A	150 TBP	150 A	Serum PBI (μg./100 ml.)	Serum total protein (gm./100 ml.)	Serum albumin (gm./100 ml.)
Non-pregnant Women											
1	81.4	9.7	55.0	36.1	47.6	46.3	37.4	53.4	4.7	6.4	3.9
2	85.6	10.5	54.2	37.4	42.5	50.6	34.1	56.7	5.0	6.9	4.0
3	81.7	10.9	51.8	40.1	42.3	50.5	33.0	58.4	5.1	7.0	4.6
4	80.5	14.0	53.3	41.8	41.5	53.1	38.3	55.4	4.9	7.2	4.5
5	80.2	13.8	49.2	45.2	34.9	57.1	22.1	71.5	5.9	6.3	3.9
6	77.6	18.4	43.1	44.9	32.9	58.1	22.0	69.6	6.0	6.4	4.0
7	82.4	13.1	53.9	39.6	41.1	51.3	28.1	64.3	5.4	7.2	4.5
8	80.1	16.3	52.3	41.8	38.1	57.0	25.2	64.6	5.2	7.1	4.0
9	74.1	21.6	39.0	52.4	25.6	66.4	19.9	71.6	5.2	7.1	4.1
Mean	80.4	14.6	50.2	42.1	38.5	54.5	28.9	62.8	5.3	6.8	4.1
Std. dev.	3.2	2.9	5.5	4.9	6.5	5.9	7.0	7.1	0.4	0.4	0.3
First Trimester (6 to 13 weeks, inclusive)											
1	90.3	6.5	79.5	15.6	64.3	28.6	43.0	49.7	7.8	5.8	3.7
2	89.2	5.0	82.5	11.2	63.9	35.9	55.6	38.8	7.3	6.9	3.2
3	90.9	5.4	78.0	15.7	65.0	30.1	56.0	38.6	10.0	6.5	3.6
4	91.2	5.3	87.8	8.9	68.6	26.2	47.5	44.5	6.9	6.7	3.3
5	91.6	5.5	81.3	15.1	65.0	30.0	41.5	48.0	6.5	6.5	3.8
6	90.0	6.5	77.4	17.8	63.0	30.6	43.0	47.0	10.0	6.0	3.6
Mean	90.5	5.7	81.1	14.0	65.0	30.2	47.8	44.4	8.1	6.4	3.5
Std. dev.	0.9	0.6	3.8	3.3	1.9	3.2	6.5	4.7	1.6	0.4	0.2
Second Trimester (14 to 27 weeks, inclusive)											
7	93.4	3.6	77.8	13.0	62.0	35.0	47.5	42.6	8.5	6.3	4.0
8	93.9	3.5	87.5	8.5	69.7	21.8	57.2	34.2	8.9	6.7	3.1
9	92.7	4.9	75.4	17.1	62.2	30.2	45.7	47.9	6.1	6.3	3.5
10	93.3	4.0	83.9	10.2	69.1	23.6	57.7	36.1	6.9	6.0	3.7
11	91.3	4.8	79.4	14.9	61.6	30.9	45.9	45.8	4.9	6.5	3.7
12	93.6	4.9	82.8	12.1	68.2	23.9	50.5	40.1	8.8	6.6	3.5
13	95.0	3.9	84.4	9.8	67.0	23.9	48.3	38.4	7.0	6.3	3.0
14	91.3	4.5	76.5	17.3	66.4	32.9	40.2	48.6	6.5	6.6	3.6
Mean	93.1	4.3	81.9	12.9	65.8	27.8	49.1	41.6	7.2	6.4	3.5
Std. dev.	1.3	0.6	4.2	3.3	3.3	5.0	5.9	5.5	1.3	0.2	0.3
Third Trimester (28 to 40 weeks, inclusive)											
15	93.7	3.5	84.2	12.3	65.6	28.8	48.3	44.9	8.8	6.3	3.6
16	95.3	4.0	89.7	7.5	72.7	21.2	52.7	39.0	10.6	6.5	3.7
17	92.4	5.2	83.2	9.8	73.3	21.0	61.0	32.8	9.5	6.3	3.7
18	87.4	3.4	86.4	6.4	71.7	21.3	52.0	39.8	7.1	6.4	2.8
19	86.5	5.0	81.6	13.8	63.0	29.2	48.6	42.9	8.2	6.3	3.1
20	87.4	7.5	82.3	15.3	61.7	28.2	43.0	46.6	8.7	6.9	3.3
21	92.8	4.6	82.2	15.4	64.6	28.6	45.7	46.7	8.7	5.5	2.8
22	89.5	6.4	70.3	21.9	54.9	46.7	39.6	50.3	7.0	6.2	3.5
Mean	90.6	4.9	82.5	12.5	65.9	26.9	48.9	42.9	8.6	6.3	3.3
Std. dev.	3.4	1.4	5.6	5.0	6.4	5.4	6.6	5.5	1.2	0.4	0.4
Entire Pregnant Group											
Mean	91.5	4.9	81.8	13.3	65.6	27.7	48.7	42.8	7.9	6.4	3.4
Std. dev.	2.4	1.2	4.5	3.5	4.2	4.5	6.0	5.1	1.4	0.3	0.3

* Based on original gravimetric addition.
† Per cent of total radioactivity associated with the thyroxine-binding protein.
‡ Per cent of total radioactivity associated with albumin.

the stained electrophoretograms were analyzed by photoelectric densitometry. For adequate detection of radioactivity in the experiments with tracer quantities of radiothyroxine and artificial "extracellular fluid" mixtures, three replicate electrophoretograms were superimposed upon one another prior to scanning in the automatic strip scanner.

Analysis of the distribution of radioactivity was per-

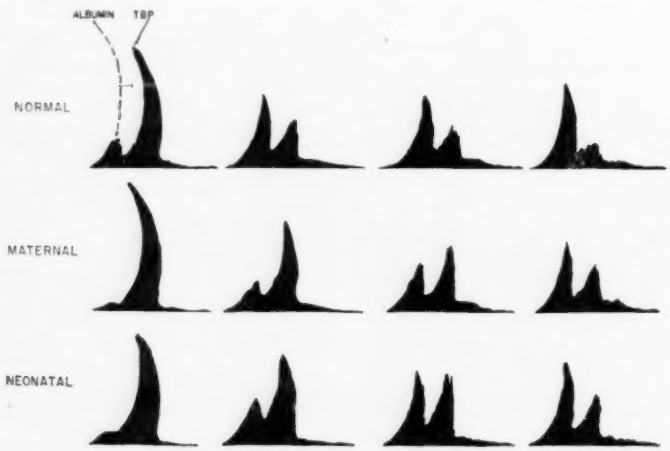

FIG. 1. COMPARISON OF SCANS OF RADIOACTIVITY OBTAINED FROM ELECTROPHORETOGRAMS OF REPRESENTATIVE CONTROL, MATERNAL, AND NEONATAL SERA AT VARIOUS CONCENTRATIONS OF ADDED THYROXINE

Concentrations of added thyroxine are, from left to right: 10, 48, 85, and 160 μg. per cent.

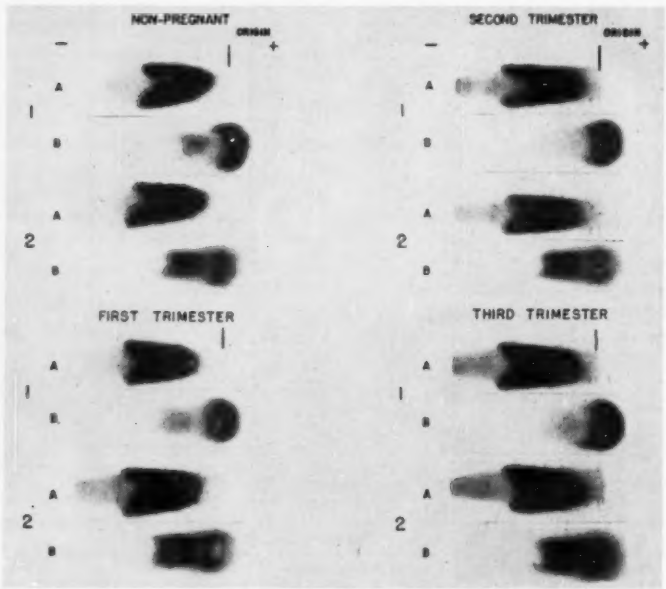

FIG. 2. ELECTROPHORETIC MIGRATION OF TBP IN SERA FROM PREGNANT AND NON-PREGNANT WOMEN AT PH 4.5

Sera were electrophoresed in Whatman 3 filter-paper at pH 4.5, acetate buffer, ionic strength 0.1 for 24 hours at 180 volts.

1. Concentration of added thyroxine 10 μg. per cent.
2. Concentration of added thyroxine 48 μg. per cent.

A. Brom-phenol blue stain.

B. Radioautograph of electrophoretogram obtained prior to staining.

formed by tracing the original graphic recordings of radioactivity obtained from each individual strip on hard paper of uniform thickness. The total curve, and the peaks representing TBP and albumin were cut from the tracings and weighed, using a semi-automatic analytic balance of 0.05 mg. sensitivity. The weights of the curves representing radioactivity bound to albumin and to TBP were expressed as percentages of the total curve weight. This technique allowed rapid and accurate integration of the curves of radioactivity obtained.

Counting rates for individual experiments ranged from 3,000 to 15,000 counts per minute, depending on the specific activity of the radioactive thyroxine used. Background counting rates averaged 16 counts per minute. A counting accuracy of 3 per cent or greater was achieved.

RESULTS

Normal pregnancy (Table I)[7]

During electrophoresis at pH 8.6 and at pH 4.5 radiothyroxine added to sera of both pregnant and non-pregnant women displayed identical mobility (Figures 1, 2). However, at pH 8.6, in sera from pregnant subjects in each of the trimesters, as well as from the pregnant group considered as a whole, and at each concentration of added thyroxine, the percentage of radiothyroxine associated with TBP was significantly greater, and the percentage of radiothyroxine associated with albumin was significantly less than corresponding values in the control sera ($P < 0.001$). Significant differences between individual trimesters could not be discerned, except in the case of the lowest concentration of thyroxine, in which TBP-bound thyroxine in the second trimester exceeded that in the first ($P < 0.05$). Poor electrophoretic separation of albumin and TBP at pH 4.5 precluded comparable quantitative analysis of the binding distribution at this pH. However, it was possible to demonstrate, at pH 4.5, that progressive displacement of radiothyroxine from TBP onto albumin occurred with increasing concentrations of added stable thyroxine (Figure 2).

[7] Data are presented in terms of the percentage of thyroxine bound to TBP and albumin at varying concentrations of added thyroxine. Since endogenous and exogenous thyroxine are in equilibrium, it is evident that the partition of radiothyroxine in serum depends upon the total concentration of both exogenous and endogenous thyroxine (24, 26). Because of the individual variation in endogenous PBI, no attempt was made to present the total thyroxine concentration in each serum following supplementation. It is apparent, however, that since PBI's are greater than normal in sera from pregnant women, the significance of observed differences in the partition of thyroxine is enhanced.

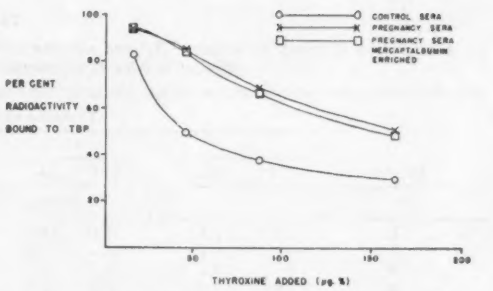

FIG. 3. PER CENT THYROXINE BOUND TO TBP AT VARIOUS CONCENTRATIONS OF ADDED THYROXINE

Comparison of sera of pregnant women before and after enrichment with mercaptalbumin with sera of non-pregnant women.

In Table II are compiled the values for per cent thyroxine bound to TBP and to albumin when all the samples of sera from the same subjects described in Table I were supplemented gravimetrically with mercaptalbumin in amounts sufficient to achieve albumin concentrations of approximately 4.6 gm. per cent. No significant difference was discernible in the percentage of thyroxine bound to either TBP or albumin in unsupplemented and supplemented sera (Figure 3). However, pure solutions of mercaptalbumin exhibited a high binding affinity for thyroxine since,

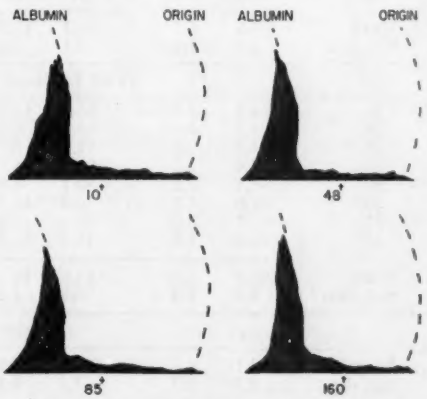

FIG. 4. THYROXINE-BINDING BY MERCAPTALBUMIN AT VARIOUS CONCENTRATIONS OF ADDED THYROXINE

Mercaptalbumin, 2.0 gm./100 ml. of 0.85 per cent saline, was electrophoresed in Whatman 3 filter-paper at pH 8.6, barbital buffer, ionic strength 0.05 for 16 hours at 120 volts.

TABLE II

*Distribution of thyroxine between TBP and albumin during filter-paper electrophoresis of sera from pregnant women (effect of in vitro supplementation of sera with mercaptalbumin *)*

Subject	Thyroxine added (μg./100 ml.)† 10 TBP‡	10 A§	48 TBP	48 A	85 TBP	85 A	160 TBP	160 A	Serum albumin (gm./100 ml.)
Non-pregnant Women									
1	89.2	7.5	55.0	38.6	41.6	49.4	32.2	57.9	4.6
2	87.3	8.7	56.6	37.8	41.9	51.3	34.6	59.1	4.5
3	81.1	13.4	47.5	47.6	34.5	59.6	22.5	71.2	4.6
4	85.4	11.9	50.0	42.2	37.6	56.4	33.3	63.7	4.6
5	80.7	13.8	48.3	45.2	35.7	58.8	26.7	66.2	4.6
6	77.5	17.8	42.0	51.1	33.5	60.5	29.6	62.4	4.6
7	82.7	17.8	42.0	51.1	33.5	60.5	29.6	62.4	4.5
8	78.5	15.5	53.9	38.8	38.7	53.7	29.1	61.8	4.5
9	78.2	17.0	44.7	50.4	34.0	58.1	25.7	67.2	4.6
Mean	82.3	13.1	50.6	43.6	43.1	54.9	29.8	62.8	
Std. dev.	3.7	3.5	5.5	5.5	4.0	5.0	4.3	4.9	
First Trimester (6 to 13 weeks, inclusive)									
1	95.0	4.1	80.1	14.9	62.9	32.4	46.8	48.8	4.6
2	84.6	2.9	89.6	19.3	57.6	38.8	44.8	50.9	4.7
3	91.6	4.6	86.6	15.4	71.5	23.3	56.9	37.8	4.6
4	89.3	5.8	78.6	17.7	63.9	31.2	44.7	48.6	4.6
5	88.1	7.2	75.9	8.8	58.3	35.8	44.3	47.1	4.6
6	87.7	6.8	77.6	14.7	62.4	30.9	47.0	44.8	4.6
Mean	90.2	5.2	81.4	15.1	62.8	32.1	47.4	46.4	
Std. dev.	2.7	1.7	5.5	3.6	4.2	5.2	4.8	4.6	
Second Trimester (14 to 27 weeks, inclusive)									
7	94.5	2.7	75.3	13.9	59.8	30.1	46.9	43.7	4.7
8	93.4	3.9	82.4	9.9	70.7	26.1	50.6	41.4	4.6
9	93.0	4.5	80.2	20.4	54.5	39.8	41.1	51.5	4.6
10	93.0	3.0	80.4	12.9	62.5	30.9	47.1	45.3	4.6
11	94.0	3.4	86.8	13.8	61.0	31.8	43.1	45.3	4.6
12	89.8	6.4	78.8	17.0	60.3	34.5	42.9	52.7	4.6
13	90.5	5.9	80.1	15.8	60.6	24.9	43.0	52.4	4.7
14	92.8	4.9	77.5	17.8	56.2	37.0	44.9	48.6	4.6
Mean	92.6	4.4	80.2	15.2	60.7	31.9	44.9	47.6	
Std. dev.	2.8	1.4	3.4	3.3	4.8	5.1	3.1	4.3	
Third Trimester (28 to 40 weeks, inclusive)									
15	94.1	3.9	83.0	11.7	65.1	29.3	46.3	46.9	4.6
16	93.3	4.0	88.2	8.4	70.8	22.6	51.5	39.9	4.7
17	94.7	3.1	91.6	5.0	79.6	15.5	56.7	31.3	4.7
18	93.6	2.5	90.7	5.1	72.9	20.4	57.4	33.4	4.5
19	95.4	2.1	87.1	8.9	67.8	24.5	47.9	41.0	4.6
20	91.0	5.1	76.6	17.8	59.8	32.7	45.8	46.9	4.6
21	92.1	5.0	74.9	18.0	60.8	33.4	41.3	48.7	4.6
22	90.4	5.7	76.6	18.0	57.4	36.1	44.7	48.6	4.6
Mean	93.1	3.9	83.6	11.6	66.8	26.8	48.9	42.1	
Std. dev.	1.8	1.3	6.8	4.2	7.5	7.2	5.8	6.9	
Entire Pregnant Group									
Mean	92.1	4.4	81.6	13.9	63.5	30.1	47.1	45.3	
Std. dev.	2.3	1.3	5.3	4.5	6.3	6.2	4.2	5.8	

* Same patients as in Table I.
† Based on original gravimetric addition.
‡ Per cent of total radioactivity associated with the thyroxine-binding protein.
§ Per cent of total radioactivity associated with albumin.

TABLE III

Distribution of thyroxine between TBP and albumin during filter-paper electrophoresis of sera from women in early pregnancy

	Thyroxine added (μg./100 ml.)*							
	10		48		85		160	
	TBP†	A‡	TBP	A	TBP	A	TBP	A
Experiment 1								
Control§	70.0	12.5	39.5	42.8	36.8	53.7	26.1	64.9
Pregnancy 21st Postovulatory day	83.5	7.0	55.3	33.3	43.3	38.5	31.6	50.6
Experiment 2								
Control§	73.5	14.0	42.6	38.6	28.9	59.8	24.0	64.8
Pregnancy 21st Postovulatory day	85.7	7.9	56.1	35.4	39.1	40.9	27.2	51.6

* Based on original gravimetric addition.
† Per cent of total radioactivity associated with the thyroxine-binding protein.
‡ Per cent of total radioactivity associated with albumin.
§ Mean values of 5 non-pregnant women.

at each of the four concentrations of thyroxine employed, at least 80 per cent of added radiothyroxine migrated with the mercaptalbumin during electrophoresis (Figure 4).

Sera obtained from 2 pregnant patients on the estimated twenty-first postovulatory day revealed percentages of thyroxine bound to TBP and to albumin approaching values found in normal pregnancy of longer duration, and differing markedly from values in concurrently electrophoresed sera from non-pregnant controls (Table III).

In serum from the three patients observed dur-

TABLE IV

Distribution of thyroxine between TBP and albumin during filter-paper electrophoresis of sera from women in the puerperium

	Thyroxine added (μg./100 ml.)*								
	10		48		85		160		Serum PBI (μg./100 ml.)
	TBP†	A‡	TBP	A	TBP	A	TBP	A	
Non-pregnant Women									
1	79.1	17.1	54.2	38.7	46.0	48.1	38.4	56.7	5.0
2	80.2	16.8	53.7	37.9	44.0	49.8	39.0	56.9	5.2
3	78.4	15.9	55.1	38.4	47.4	47.6	37.9	55.1	4.8
Puerperal Subject 1									
Term	95.0	3.4	89.8	7.2	69.4	23.7	52.4	42.4	9.0
1 Week	94.2	4.8	78.1	18.1	60.5	38.4	47.9	47.4	8.4
3 Weeks	92.6	5.1	70.8	20.9	56.8	41.4	45.5	48.3	6.6
5 Weeks	88.7	8.4	63.7	29.8	51.3	46.6	42.1	50.1	5.9
Puerperal Subject 2									
Term	95.1	2.8	85.2	10.9	69.1	21.6	59.2	32.2	8.5
1 Week	94.0	5.1	77.6	16.8	59.2	31.8	52.2	31.7	7.8
3 Weeks	94.5	3.6	72.6	21.4	60.1	28.0	49.1	38.4	5.8
5 Weeks	91.6	5.6	65.4	26.4	50.8	40.9	41.5	43.7	5.5
Puerperal Subject 3									
Term	94.4	4.0	87.1	8.4	70.2	22.4	60.0	29.8	7.7
1 Week	92.3	5.1	76.4	16.9	58.1	32.8	48.0	44.7	6.9
3 Weeks	87.9	7.2	77.1	14.1	52.6	34.7	44.8	47.5	6.0
5 Weeks	84.8	9.1	69.2	21.7	52.3	37.8	41.9	50.6	4.6

* Based on original gravimetric addition.
† Per cent of total radioactivity associated with the thyroxine-binding protein.
‡ Per cent of total radioactivity associated with albumin.

ing the puerperium, thyroxine-binding by TBP was approaching, but had not yet returned to normal non-pregnant values by the end of the fifth post-partum week (Table IV).

Despite considerable variation, concentrations of SPI were significantly greater than normal during the first and subsequent trimesters. Significant differences between individual trimesters were also noted, the mean of third trimester concentrations of SPI being the highest and the mean of the second trimester values being the lowest (Table I). The concentrations of SPI in the puerperium decreased to control values by the fifth post-partum week (Table IV).

Except in the first trimester, the concentrations of total protein in the sera obtained from pregnant women were slightly lower than those of the controls ($P < 0.02$). Significant reduction in the concentration of albumin in sera from normally pregnant women was apparent in all three trimesters ($P < 0.01$) (Table I).

Thyroxine-binding by TBP in serum from all of the 6 patients with threatened or inevitable abortion was not assayed concurrently. Therefore, in order to intercompare results among the 6 patients, the percentage of thyroxine bound to TBP in these patients has been expressed as a percentage of the corresponding values in concurrently analyzed sera from normal, non-pregnant controls (Table V). These values were then compared with comparably calculated percentages for pregnant women in the first trimester (derived from the data in Table I). The results then fell into two categories. In sera from four of the patients, alterations in the partition of thyroxine of a degree characteristic of normal pregnancy were not observed. These patients either aborted dead fetuses spontaneously or, following development of negative A-Z tests and withdrawal of the blood sample to be analyzed, underwent dilatation and curettage for incomplete abortion. Two of the 6 patients revealed changes in the partition of thyroxine consistent with normal pregnancy of similar duration. In spite of vaginal bleeding sufficient to warrant hospitalization with a diagnosis of threatened abortion, one patient is in her ninth month of apparently normal pregnancy and the other recently underwent normal parturition.

Table VI and Figure 1 present data obtained during a single experiment comparing sera obtained at term, sera of the corresponding newborn, and sera from non-pregnant controls. Neonatal concentrations of SPI (7.9 ± 0.8 μg. per cent) (mean $\pm$ standard deviation) were significantly greater than control values ($P < 0.05$), but did not differ significantly from maternal concentrations (8.5 ± 0.5 μg. per cent). In maternal sera, all values of the percentage of thyroxine bound

TABLE V

Distribution of thyroxine between TBP and albumin during filter-paper electrophoresis of sera from patients with a diagnosis of threatened or inevitable abortion *

	Thyroxine added (*µg./100 ml.*)†				Serum PBI (*µg./100 ml.*)	Serum total protein (*gm./100 ml.*)	Serum albumin (*gm./100 ml.*)	Week of pregnancy
	10	48	85	160				
				Control				
	100.0	100.0	100.0	100.0	5.3	6.8	4.1	
				Pregnancy (First Trimester)				
	112.5	160.9	145.7	159.1	8.1	6.4	3.5	11‡
				Threatened or Inevitable Abortion				
1	100.0	118.0	109.0	110.4	5.5	6.5	3.8	10
2	100.0	96.9	115.4	115.0	4.8	7.0	4.2	13
3	122.8	121.5	111.3	98.9	6.0	7.1	4.4	12
4	110.2	114.9	98.1	101.4	5.8	7.0	3.8	15
5	120.6§	147.4	134.7	174.5	5.8	7.1	4.2	5
6	130.4§	168.8	169.7	214.5	7.7	6.8	4.3	12

* Percentage of radioactivity associated with TBP at varying concentrations of stable thyroxine expressed as per cent of control values for the individual experiment.

† Based on original gravimetric addition.

‡ Mean duration of pregnancy in first trimester subjects of Table I.

§ Pregnancy not interrupted in these cases.

TABLE VI

Distribution of thyroxine between TBP and albumin during filter-paper electrophoresis of sera from pregnant women at term and from their respective newborn

Subject	Thyroxine added (μg./100 ml.)* 10 TBP†	10 A‡	48 TBP	48 A	85 TBP	85 A	160 TBP	160 A	Serum PBI (μg./100 ml.)	Serum total protein (gm./100 ml.)	Serum albumin (gm./100 ml.)
Non-pregnant Women											
1	70.3	23.5	36.8	54.5	27.7	62.7	18.2	71.6	5.0	8.0	4.8
2	74.0	20.0	42.1	49.6	32.4	61.0	22.4	67.7	5.1	7.1	4.0
3	79.7	17.7	40.2	48.3	34.3	51.8	25.7	60.6	5.0	6.9	4.0
4	76.7	18.9	46.3	44.0	33.1	54.7	23.9	65.5	5.9	6.3	3.9
5	77.8	16.3	51.6	39.4	41.4	47.6	33.6	52.0	5.4	7.2	4.5
6	71.1	22.1	39.6	53.0	31.5	58.4	24.3	64.6	4.6	7.0	4.2
7	83.7	10.1	55.0	33.2	40.7	53.9	35.3	56.3	5.0	6.7	4.4
8	72.3	24.8	38.0	59.6	30.0	63.4	25.8	67.2	6.0	6.4	4.0
Mean	75.7	19.2	43.7	47.7	33.9	56.7	26.2	63.2	5.3	6.9	4.2
Std. dev.	4.5	4.6	6.6	8.5	4.8	5.6	5.7	6.5	0.5	0.5	0.3
Maternal, at Term											
1	95.2	3.1	75.1	11.2	58.3	30.5	46.1	42.3	8.6	6.2	3.3
2	91.0	5.7	68.7	22.6	50.0	34.1	39.8	44.1	9.0	6.0	3.3
3	93.0	3.2	74.1	23.6	58.4	30.6	46.9	40.7	9.5	6.4	3.4
4	96.2	1.8	86.7	8.3	68.8	21.5	58.7	34.4	8.4	6.0	2.7
5	95.6	1.6	90.2	5.9	71.3	20.3	61.1	28.2	7.8	6.7	3.4
6	93.9	3.7	86.7	8.9	72.8	22.8	51.4	44.9	8.5	6.4	3.5
7	86.3	3.5	82.9	9.9	68.7	21.6	54.1	34.0	8.2	6.0	3.9
8	94.5	3.4	82.8	11.7	66.0	29.1	51.5	42.9	8.0	6.2	3.3
Mean	93.2	3.3	80.9	12.8	64.3	26.3	51.2	38.9	8.5	6.2	3.3
Std. dev.	3.2	1.3	7.5	6.6	7.9	5.3	6.9	6.0	0.5	0.3	0.3
Corresponding Newborn											
1	91.5	6.0	67.7	27.6	47.1	44.7	34.3	54.4	9.0	6.4	3.4
2	98.3	7.5	65.5	29.0	45.0	45.1	37.9	56.0	9.2	7.1	3.4
3	89.4	7.5	62.3	30.8	52.3	36.2	47.2	43.1	6.7	5.4	3.4
4	97.1	2.5	75.0	20.8	59.8	34.5	49.2	42.6	7.5	6.1	3.3
5	90.8	3.9	80.5	15.2	55.1	35.5	52.4	36.6	8.3	5.7	3.5
6	92.1	4.2	63.8	33.5	57.0	37.0	40.3	53.0	7.0	6.2	3.4
7	88.4	5.4	68.5	25.3	52.9	42.0	40.9	53.8	7.6	6.2	3.8
8	93.2	5.1	68.2	30.7	49.6	43.9	44.4	48.4	7.8	5.7	3.3
Mean	91.5	5.3	68.9	26.6	52.4	39.9	43.3	48.5	7.9	6.1	3.4
Std. dev.	2.8	1.7	6.0	6.0	5.0	4.5	6.1	7.0	0.9	0.5	0.2

* Based on original gravimetric addition.
† Per cent of total radioactivity associated with the thyroxine-binding protein.
‡ Per cent of total radioactivity associated with albumin.

TABLE VII

Distribution of thyroxine between TBP and albumin during filter-paper electrophoresis of dilute sera

	TBP*	A†	PBI‡ (μg./100 ml.)	Total protein‡ (gm./100 ml.)	Albumin‡ (gm./100 ml.)
Non-pregnant Control					
Undilute serum	80.3	10.5	5.6	6.4	4.4
Dilute serum	48.6	27.1	2.2	1.4	1.1
Pregnant					
Undilute serum	89.9	4.2	8.1	5.6	4.0
Dilute serum	70.4	13.9	2.3	1.3	1.0

* Per cent of total radioactivity associated with the thyroxine-binding protein.
† Per cent of total radioactivity associated with albumin.
‡ Chemically-determined composition of the final mixtures which were electrophoresed on filter-paper. For details of the preparation of these mixtures, see text.

to TBP were significantly greater than corresponding values among controls ($P < 0.001$). In the case of the newborn, a similar difference existed ($P < 0.01$). However, the percentage of thyroxine bound to TBP in neonatal sera was significantly less than that bound to TBP in maternal sera, except at the lowest concentration of added thyroxine employed.

The concentrations of total protein in the neonatal sera (6.1 ± 0.5 gm. per cent) were not significantly different from the values in the maternal sera (6.2 ± 0.3 gm. per cent), nor were the concentrations of albumin in neonatal sera (3.4 ± 0.2 gm. per cent) significantly greater than those values in maternal sera (3.3 ± 0.3 gm. per cent). Concentrations of total protein and albumin in maternal and neonatal sera were significantly less than control values ($P < 0.01$). Differences in the composition of sera from non-pregnant women, from pregnant women at term, and from neonatal infants are represented in the densitometric scans depicted in Figure 5.

Table VII depicts one of four experiments in which sera were diluted tenfold and then supplemented with sufficient albumin and radiothyroxine to simulate the concentrations of total protein and PBI which are reportedly present in extracellular fluid (27). In all of these studies, an enhanced TBP-activity was exhibited by artificial "extracellular fluid" mixtures prepared from sera of normally pregnant women.

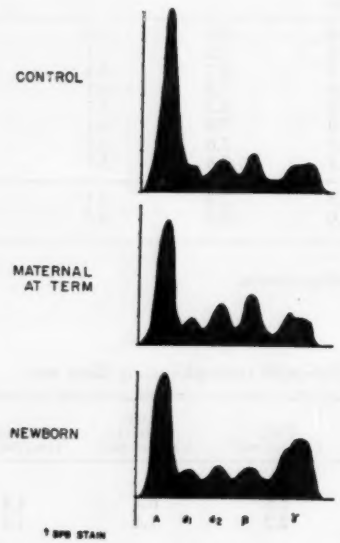

FIG. 5. PHOTOELECTRIC DENSITOMETRY APPLIED TO ELECTROPHORETOGRAMS, STAINED WITH BROM-PHENOL BLUE, OF CONTROL, MATERNAL, AND NEONATAL SERA

Sera were electrophoresed in Whatman 3 filter-paper at pH 8.6, barbital buffer, ionic strength 0.05 for 16 hours at 120 volts. Curves represent average values of optical density from densitometry applied to all electrophoretograms of the experiment comparing the sera described.

DISCUSSION

When thyroxine is added to plasma, it partitions among the plasma proteins according to their relative binding affinities for this hormone. At physiological concentrations, thyroxine in plasma is principally associated with a protein (or proteins) which, at pH 8.6, displays an electrophoretic mobility intermediate to that of the alpha-1 and alpha-2 globulins (7–11). This globulin moiety has been designated the thyroxine-binding protein of plasma (TBP). As the concentration of thyroxine in plasma is increased, a progressively larger fraction of the hormone is bound to albumin, its chief secondary carrier (26, 28, 29, 24).

In the present communication, it has been demonstrated that similar qualitative relationships obtain in maternal serum during electrophoresis at pH 8.6 and pH 4.5. Measurement of the partition of thyroxine, in several standardized concentrations, between TBP and albumin affords a convenient means for intercomparing the thyroxine-binding activity of TBP in individual sera. By means of this technique, a marked augmentation has been found in the thyroxine-binding capacity of TBP in sera obtained from normally pregnant women. This augmentation begins as early as the twenty-first postovulatory day, persists throughout pregnancy, and into the puerperium.

In confirmation of earlier reports, moderate hypoalbuminemia has been found in normally pregnant women (14–16). Since the distribution of thyroxine between albumin and TBP *in vitro* is in part dependent on the concentration of these proteins, it seemed possible that either physiological hypoalbuminemia or some alteration in the binding properties of maternal albumin might explain the unusual avidity for thyroxine of TBP in maternal sera. This did not prove to be the case. Correction of hypoalbuminemia by the *in vitro* addition of an albumin which exhibited considerable thyroxine-binding potency, and which had been

obtained from multiple non-pregnant donors, did not demonstrably alter the distribution of thyroxine among the proteins of maternal sera.

The above observations concern the partition of thyroxine among the proteins of plasma under circumstances in which the total concentration of thyroxine has been increased approximately 100 per cent during the addition of radioactive hormone. At truly physiological concentrations of thyroxine, so small a fraction of radiothyroxine is bound to albumin that assessment of a possible increase in TBP-activity of maternal sera is technically unfeasible. However, when truly tracer amounts of radiothyroxine were employed, and sera were diluted and supplemented with albumin so that concentrations both of total protein and of PBI approached those reportedly present in extracellular fluid (27), enhancement of TBP-activity in maternal sera as compared to controls could once again be demonstrated.

Since direct measurement of the concentration of TBP in serum cannot yet be made, it is not possible to ascertain whether the present findings result from an absolute increase at the quantity of TBP in the serum of pregnant women or from an alteration in those molecular characteristics of TBP which determine its affinity for thyroxine.

Although the enhanced TBP activity in sera from pregnant patients is as yet unexplained, several observations suggest that hormonal changes occurring during pregnancy may be implicated. The initial appearance and the subsequent post-partum disappearance of augmentations both in SPI and in binding capacity of TBP are temporally well correlated. Secondly, Engstrom, Markardt, and Liebman have demonstrated that large doses of estrogen are capable of inducing augmentation of the SPI in males and non-pregnant females (30, 31). Finally, early and continued increase in the elaboration of estrogens occurs during the pregnant state (32). For these reasons, diethylstilbestrol, in doses of 30 mg. daily, has been administered to a group of non-pregnant females. Preliminary data indicate that this regimen induces, within 4 weeks, an increase in the thyroxine-binding capacity of TBP similar to that seen during pregnancy. It would thus appear that increased elaboration of estrogen during pregnancy may be at least in part responsible for the phenomena reported above. However, the extent to which other hormonal or non-hormonal factors may be contributory remains to be determined.

In this regard it is perhaps noteworthy that among 6 patients with spontaneous abortion, 4 failed to demonstrate alterations in TBP of the magnitude observed in normal pregnancy. In 2 patients with threatened abortion, augmentations of thyroxine-binding by TBP similar to those seen during normal pregnancy were found. One of these patients is in her ninth month of apparently normal pregnancy, and the other recently underwent normal parturition. It is unlikely that failure to demonstrate the usual gravid change in thyroxine-binding can be explained by recent death of the fetus, since following normal pregnancy increased thyroxine-binding is evident for as long as 5 weeks post partum. In the abnormal situation, it appears more likely that the usual gravid changes had either failed to appear or that the stimuli responsible for their production had been withdrawn a considerable period before actual abortion. Whether this sequence reflects inadequacies of implantation and trophoblastic function originating in the conceptus, or inadequacies in the maternal response to normal pregnancy remains undetermined.

Whether thyroxine crosses the placental barrier in humans is unknown. In confirmation of earlier reports (13, 33), SPI's in maternal and cord blood, although not identical, were not, in the present study, significantly different. A free passage of all proteins between maternal and fetal circulations is unlikely, since with the exception of gamma globulin, the electrophoretic components of serum obtained from cord blood resemble those of the non-pregnant adult more nearly than those of the mother (34). Nevertheless, it has been demonstrated that various gamma globulins pass from the maternal to the fetal circulation (35). In the present study, the thyroxine-binding capacity of TBP in neonatal serum has been shown to exceed that of the non-pregnant adult. It is unknown at present whether this reflects transplacental passage of TBP, a fetal response conditioned by those factors which induce alterations of maternal TBP, or an innate characteristic of the newborn.

The physiological role of the thyroxine-binding protein is unknown. It is not possible, therefore, to evaluate with certainty the relation of the increased binding capacity of TBP to the other al-

terations in the metabolism of iodine which occur during pregnancy. However, certain observations concerning the effects of TBP on the metabolism of thyroxine by tissue slices afford a basis for a preliminary hypothesis. Older observations of increase during pregnancy in number and size of thyroidal follicles, hyperplasia of follicular epithelium, and increased vascularity provide anatomical evidence of thyroidal hyperfunction. Recently, Ferraris and Scorta, studying 22 pregnant women, found an augmentation in thyroidal accumulation of I^{131} appearing in the first trimester, persisting throughout pregnancy, and returning to normal by the thirtieth post-partum day. Twenty-four-hour plasma I^{131} conversion ratios were also increased (3). Earlier, Pochin had described augmented accumulation of radioiodine in 5 pregnant women (2). These data indicate that unless dietary iodine is diminished, there occur during pregnancy increased glandular production and release of iodinated materials. Furthermore, evidence obtained by butanol-extraction and chromatographic techniques indicates that thyroxine is the principal iodinated compound contributing to the augmented SPI of pregnancy (3, 12, 13). Thus, there is evidence during pregnancy of the thyroidal production and release of increased quantities of calorigenically potent hormone.

However, in spite of this increase in the production and circulating concentration of thyroid hormone, there is no associated augmentation of basal energy requirements beyond those which may be attributable to the needs of the fetus. Pregnancy thus appears to disrupt the usual relationship between augmented concentration of circulating thyroid hormone and cellular metabolic response. Further evidence of a dissociation of this nature is provided by the amelioration of thyrotoxicosis and reduction in requirement for antithyroid drug, often observed in thyrotoxic pregnant patients (36–38). Humoral factors during pregnancy might reduce metabolic responsiveness of tissues to thyroid hormone or directly reduce respiration in tissues (39). However, this possibility is rendered unlikely by the observation that ameliorated thyrotoxicosis may not exacerbate until 9 or 10 weeks post partum (40), long after evidence of increased secretion of at least estrogenic and adrenocortical hormones has disappeared (32, 41). Furthermore, in normal women, hyperthyroidism does not appear during that portion of the puerperium in which estrogenic alterations have regressed and SPI remains increased.

If tissue responsivity is indeed undiminished, then the majority of maternal tissues must receive at most a normal quantity of hormone, despite the increase in SPI. There is at present no direct evidence that TBP participates in producing this alteration in proportional cellular penetration. Nevertheless, data obtained in an *in vitro* system suggest that it may. *In vitro,* the static distribution of thyroxine between extracellular and cellular phases is conditioned by the relative abundance of extracellular and cellular thyroxine-binding components (42). Thus, in pregnancy, enhanced extracellular TBP could provide for the passage of only normal quantities of thyroxine to most maternal tissues, and could, in this way, protect them from the usual metabolic effects of increased concentration of circulating thyroid hormone. Increased amounts of thyroxine might be directed to particular maternal tissues, such as uterus, placenta, or perhaps fetus, whose requirements for hormone may be augmented, or alternatively might be channeled to sites in which hormonal degradation is not linked to metabolic action. This could be accomplished if the binding potential of these tissues favored the ready passage of thyroxine from TBP to cellular binding sites.

However, findings in the static system need not reflect the role of TBP in the dynamically equilibrated living system. *In vivo,* thyroxine may enter the cell in association with TBP. If this were true, delivery of thyroxine to tissues would be limited by the transcellular flux or cellular degradation of TBP. If, then, during pregnancy, thyroxine and TBP are increased proportionately, so that their molecular ratio remains constant, continued passage of normal amounts of TBP into the majority of maternal tissues would provide for the cellular penetration of normal quantities of thyroxine. Furthermore, the needs of certain tissues with increased hormonal requirements, such as those noted above, could be met by the intracellular passage of increased amounts of TBP. Under either of these circumstances, overall fractional rate of turnover of thyroxine dur-

ing pregnancy might increase, decrease, or might, as suggested by the data of Danowski, Schwartz, Rhodes, Yoder, Puntereri, and Mateer, remain normal (43).

It is apparent that the foregoing correlations are largely conjectural, being based on the limited data available, and omitting from consideration such variables as the number and avidity for thyroxine of cellular binding sites. Nevertheless, they appear to afford, at least, a conceptual frame of reference for future investigation of those puzzling alterations of thyroidal economy which occur during pregnancy.

SUMMARY

The partition of thyroxine between albumin and the specific thyroxine-binding protein (TBP) has been compared in sera obtained from non-pregnant and pregnant women. At all concentrations of thyroxine studied, a greater percentage was bound to TBP in sera of normally pregnant women than in sera of controls. Data obtained from supplementation of maternal sera with mercaptalbumin suggest that the observed alteration in thyroxine-binding does not result from the physiological hypoalbuminemia of pregnancy. Alteration in thyroxine-binding begins early in pregnancy and persists well into the puerperium. A similar though less marked augmentation of thyroxine-binding by TBP has been noted in sera obtained from umbilical cord bloods. Several patients with a diagnosis of threatened or inevitable abortion failed to demonstrate a change in thyroxine-binding capacity of TBP of the magnitude seen in normal pregnancy of similar duration.

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RENAL CIRCULATORY DYNAMICS AND URINARY PROTEIN EXCRETION DURING INFUSIONS OF *l*-NOREPINEPHRINE AND *l*-EPINEPHRINE IN PATIENTS WITH RENAL DISEASE [1, 2]

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That hemodynamic adjustments within the kidney may modify the rate of transport of protein molecules across glomerular capillary membranes has been suggested by a variety of experimental studies. The mechanisms which alter protein transport under these conditions are not clear, but since proteins apparently traverse capillary walls by diffusion as well as filtration (2), differing mechanisms may play a role depending on the nature and circumstances of the associated vascular adjustments. Thus filtration may be modified under conditions which alter intraglomerular hydrostatic pressure (3–5) and diffusion under circumstances which influence the rate of formation of glomerular filtrate (2). It has been suggested that protein transport and urinary protein excretion may be altered by renal circulatory adjustments which modify the area available for transport within the kidney (6) or which influence the integrity of the capillary wall (7–9). Moreover, it has recently been suggested that protein transport may also be conditioned by the velocity of blood flow through the glomerulus (10).

In the present study an examination of these factors was undertaken in patients with renal disease and proteinuria. The urinary excretion and renal clearance of plasma proteins were determined under circumstances in which intrarenal circulatory adjustments were induced by the administration of the adrenal medullary hormones, *l*-norepinephrine and *l*-epinephrine.

METHOD

The effects of *l*-norepinephrine on the renal clearances of inulin, sodium p-aminohippurate (PAH) and plasma proteins were determined in 16 patients, and the effects of *l*-epinephrine were determined in 7 patients, with well-documented chronic renal disease (Table I). The subjects, who varied in age from 19 to 64 years, were all males with the exception of E. Z. All had a consistently demonstrable proteinuria, ranging from a trace to 4 plus, as determined by the sulfosalicylic acid test. All studies were made in fasting subjects after the ingestion of 500 ml. of water approximately one hour prior to the test. After the subjects had been lying quietly in bed for at least one hour three control clearance periods of 10 to 15 minutes each were obtained. The arterial blood pressure, measured sphygmomanometrically, and radial pulse rate were recorded at frequent intervals during the control observations. Upon completion of the control measurements an intravenous infusion of *l*-norepinephrine or *l*-epinephrine was started. *L*-norepinephrine, made up in 5 per cent dextrose (8 μg. per ml.), was administered at a rate sufficient to elevate the systolic blood pressure 20 to 50 mm. Hg. The amount required for this purpose varied from 3 to 72 μg. per minute and usually required 15 to 20 μg. per minute. After the blood pressure, which was measured at one to two-minute intervals, had stabilized at the desired level for approximately 15 minutes, the urine was discarded and three 10 to 15-minute clearance periods were obtained during the pressor response to *l*-norepinephrine. In 8 subjects three 10 to 15-minute clearance periods were collected immediately after the infusion of *l*-norepinephrine had been discontinued.

The design of the experiments in which the effects of *l*-epinephrine [4] were studied was similar to that employed in the study of *l*-norepinephrine except that *l*-epinephrine was administered at a rate insufficient to elevate the arterial blood pressure significantly. This agent was made up in 5 per cent dextrose in a concentration of 2 μg. per ml. and was infused at a rate of from 2 to 6 μg. per minute. Recovery periods were obtained in 4 of these subjects after cessation of the infusion.

Glomerular filtration rate was measured as the inulin clearance (11) and renal plasma flow as the PAH

[1] This study was supported by a grant-in-aid from the National Heart Institute, U. S. Public Health Service, and from the American Heart Association and Greenwich Health Association.

[2] The results of this study have appeared in part in abstract form (1).

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[4] Supplied by Parke, Davis & Co., Detroit, Mich.

TABLE I

Renal hemodynamics and urinary protein excretion during infusion of adrenal medullary hormones *

Subject Diagnosis Age	S.A. M^2	BUN mgm. %	Procedure	B.P. mm. Hg	P.R. per min.	T.P. gm. %	Hct. %	C_{in} ml./min.	C_{pah} ml./min.	FF %	$U_{pr}V$ mgm./min.	C_{pr} ml./min.	C_{pr}/C_{in} $\times 10^{-3}$
						l-Norepinephrine							
R. H.	1.78	15	Control	124/76	68	5.00	42.5	136	735	18.8	1.1	0.023	0.17
Chronic			Infusion	176/104	56	5.25	42.3	127	469	27.1	16.6	0.316	2.49
glom. neph.			Recovery	130/78	76	5.10	42.6	147	615	23.9	5.1	0.100	0.68
27													
T. P.		15	Control	120/80	96	4.90	47.0	133	672	19.8	1.6	0.033	0.25
Amyloid			Infusion	144/100	72	5.10	47.7	127	519	24.5	3.3	0.065	0.51
19													
J. D.	1.74	17	Control	122/72	68	5.70	44.1	125	595	21.0	0.3	0.006	0.05
Tbc.			Infusion	160/80	62	5.60	45.6	131	495	26.5	0.5	0.010	0.08
52			Recovery	124/70	68	5.60	45.0	118	550	21.5	0.3	0.006	0.05
A. B.	1.88	14	Control	128/70	60	6.00	45.7	106	505	21.0	0.6	0.009	0.09
Chronic			Infusion	170/90	48	6.10	48.3	83	236	35.2	1.6	0.025	0.30
glom. neph.			Recovery	130/60	72	6.00	47.2	127	567	22.4	1.5	0.024	0.19
23													
A. McG.	1.72	18	Control	94/60	64	6.42	45.2	103	503	20.5	0.4	0.006	0.06
Chronic			Infusion	130/88	56	7.05	48.4	90	306	29.4	0 8	0 011	0.12
glom. neph.			Recovery	100/60	80	6.50	45.1	90	478	18.8	0.7	0.010	0.11
23													
A. K. (A)	1.70	19	Control	106/80	84	5.60	52.9	94	218	44.0	0.9	0.017	0.18
Amyloid			Infusion	150/86	74	6.45	57.2	89	133	66.0	4.0	0.063	0.71
59													
J. O'B.	1.58	19	Control	90/60	120	4.17	46.7	86	310	27.8	9.9	0.238	2.77
Amyloid			Infusion	130/80	100	4.30	48.9	91	239	38.0	16.1	0.376	4.13
46													
T. D.	1.94	16	Control	120/92	70	5.00	46.6	86	445	19.3	2.1	0.043	0.50
Chronic			Infusion	154/100	60	5.55	47.8	76	296	25.7	11.8	0.212	2.78
glom. neph.													
31													
P. L. (A)	2.06	18	Control	120/90	86	5.40	43.3	69	212	32.6	1.6	0.030	0.44
Chronic			Infusion	140/100	80	5.65	44.2	68	178	38.2	2.8	0.049	0.72
pyelo.			Recovery	128/90	84	5.45	42.9	70	214	32.7	2.5	0.046	0.66
60													
E. Z.	1.60	25	Control	122/60	82	4.70	37.7	68	496	13.7	4.8	0.103	1.52
Kimmel-			Infusion	170/80	88	4.60	37.6	66	363	18.2	10.5	0.206	3.12
Wilson													
56													
J. C.	1.60	19	Control	110/78	84	8.52	36.8	64	227	28.2	0.3	0.003	0.05
Chronic			Infusion	162/102	52	9.05	40.4	65	193	33.6	0.5	0.005	0.08
pyelo.													
25													
J. B.	1.82	22	Control	140/100	68	7.40	40.0	49	184	26.4	0.3	0.004	0.08
Chronic			Infusion	174/120	60	7.45	41.5	44	200	22.0	0.3	0.003	0.07
glom. neph.													
37													

* All values are averages of three determinations. *L*-norepinephrine or *l*-epinephrine was administered intravenously during the infusion periods. The recovery periods were obtained immediately after the infusion was discontinued. Abbreviations are as follows:

S.A. = body surface area (M^2).
BUN = blood urea nitrogen.
B.P. = arterial blood pressure.
P.R. = pulse rate.
T.P. = total serum protein concentration.
Hct. = hematocrit of venous blood.
C_{in} = inulin clearance.
C_{pah} = sodium p-aminohippurate clearance.
FF = renal filtration fraction (C_{in}/C_{pah}).
$U_{pr}V$ = urinary protein excretion.
C_{pr} = protein clearance.
C_{pr}/C_{in} = protein-inulin clearance ratio.

TABLE I—*Continued*

Subject Diagnosis Age	S.A. M^2	BUN mgm. %	Procedure	B.P. mm. Hg	P.R. per min.	T.P. gm. %	Hct. %	C_{in} ml./min.	C_{pah} ml./min.	FF %	$U_{pr}V$ mgm./min.	C_{pr} ml./min.	C_{pr}/C_{in} $\times 10^{-4}$
						l-Norepinephrine—*Continued*							
W. E. Amyloid 59	1.52	34	Control	90/60	64	4.20	35.6	35	95	36.9	1.3	0.032	0.92
			Infusion	120/74	66	4.25	39.8	29	68	41.6	2.5	0.059	2.04
			Recovery	90/60	70	4.00	35.0	41	120	34.2	2.9	0.072	1.75
E. B. Nephro-sclerosis 64	1.56	30	Control	172/84	80	6.30	43.9	34	159	21.4	0.4	0.006	0.18
			Infusion	198/90	72	6.40	44.1	38	172	22.1	0.5	0.007	0.18
J. H. Chronic pyelo. 63	1.63	52	Control	134/80	80	5.40	43.9	32	105	30.4	4.9	0.091	2.84
			Infusion	160/94	84	5.45	45.3	16	49	32.6	4.6	0.084	5.25
			Recovery	142/80	84	5.40	43.5	36	125	28.8	8.0	0.148	4.12
P. B. Polycystic 54	2.06	58	Control	166/94	72	6.10	34.8	16	62	25.8	0.9	0.014	0.88
			Infusion	190/94	64	6.05	35.9	13	57	22.8	0.7	0.012	0.93
			Recovery	160/92	68	5.60	33.6	15	71	21.2	0.9	0.016	1.07
						l-Epinephrine							
A. K. (B) Amyloid 59	1.70	15	Control	120/70	100	6.40	57.0	103	264	39.0	1.3	0.020	0.19
			Infusion	126/70	96	6.55	55.7	112	214	52.4	2.1	0.032	0.29
P. L. (B) Chronic pyelo. 60	2.06	19	Control	114/78	56	6.10	45.5	66	262	25.2	2.7	0.045	0.68
			Infusion	124/74	56	6.65	47.5	69	216	32.3	5.9	0.089	1.29
			Recovery	126/78	56	6.00	45.8	68	227	30.4	4.5	0.076	1.12
O. B. Nephro-sclerosis 63	1.86	20	Control	150/110	108	6.60	49.8	59	132	44.6	4.2	0.064	1.08
			Infusion	156/100	108	6.65	49.8	37	112	33.0	3.5	0.052	1.41
S. R. Chronic pyelo. 62	1.80	24	Control	130/80	84	5.85	43.2	57	193	29.9	1.6	0.027	0.47
			Infusion	130/70	92	5.75	44.2	44	153	28.8	1.6	0.028	0.64
L. K. Chronic glom. neph. 37	2.17	21	Control	148/110	60	5.85	48.7	51	239	21.3	1.0	0.017	0.33
			Infusion	140/100	72	5.90	49.2	47	172	27.3	1.2	0.021	0.45
			Recovery	136/100	68	5.95	48.9	49	196	25.0	1.3	0.022	0.45
J. M. Chronic pyelo. 58	1.80	34	Control	130/72	68	6.00	37.7	43	183	23.5	0.7	0.012	0.29
			Infusion	124/70	68	6.05	37.9	38	160	23.8	0.7	0.012	0.32
			Recovery	122/70	68	5.70	36.1	37	176	21.0	0.7	0.012	0.32
J. R. Nephro-sclerosis 60	1.76	47	Control	250/160	84	6.40	49.6	23	100	23.0	3.6	0.057	2.48
			Infusion	250/140	96	6.90	50.8	9	85	10.6	2.7	0.040	4.44
			Recovery	254/158	88	6.10	47.8	23	118	19.5	3.1	0.052	2.26

clearance (11). The concentration of protein in serum and urine was determined by the biuret method (12). In 16 subjects serum and urinary protein patterns were determined by paper electrophoresis. In order to increase the protein concentration of urine to levels sufficient to permit accurate evaluation by electrophoresis, aliquots of pooled urine collected during the control, infusion and recovery periods were dialyzed at a temperature of 4° C. in Visking cellophane bags against 12 per cent Dextran. Dialysis was continued until the concentration of protein in the urine approximated 60 mgm. per ml. Ten-μl. aliquots of pooled, concentrated urine and serum were applied to Whatman No. 3 filter paper and electrophoresis was carried out in a Durrum-type cell at 110 volts and 6 mA (for 8 strips) for a period of 16 hours. Diethylbarbituric acid-sodium barbital buffer of pH 8.6 and ionic strength 0.1 was employed. The paper strips were stained with bromphenol blue and the relative propor-

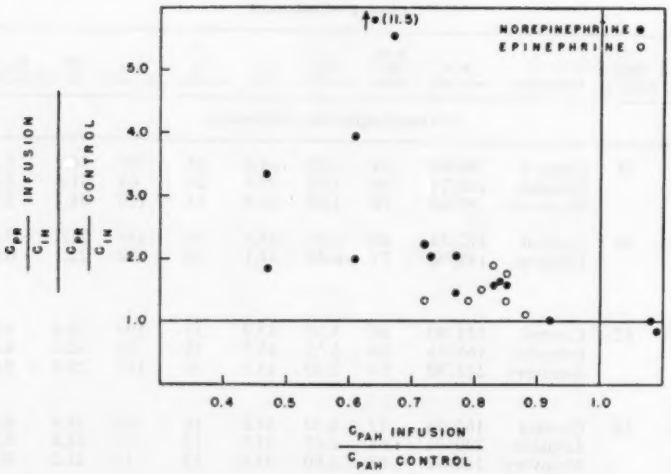

FIG. 1. RELATIONSHIP OF THE ALTERATIONS IN THE PROTEIN-INULIN CLEARANCE RATIO TO THE CHANGES IN PAH CLEARANCE DURING INFUSIONS OF *l*-NOREPINEPHRINE AND *l*-EPINEPHRINE

The protein-inulin clearance ratio during the infusion of adrenal medullary hormones relative to control values is plotted against the PAH clearance during infusion of these agents relative to control values. As the PAH clearance decreased below 85 per cent of control levels, protein clearance tended to rise relative to filtration.

tions of the individual protein fractions were determined by dye-elution. The filter paper was cut between each protein fraction at the point of lowest dye concentration as judged by the unaided eye and each fraction was eluted with 0.01 N NaOH for 30 minutes. After elution the optical density of the eluate was determined in a Coleman Universal spectrophotometer at a wavelength of 575 mμ. The amount of sodium hydroxide used was adjusted so that the optical densities of the respective fractions of the control, infusion and recovery periods were approximately equal. When determined in this manner the error of the method for measurement of the individual protein fractions was: for albumin, 1.9 per cent; α_1 globulin, 4.2 per cent; α_2 globulin 2.9 per cent; β globulin, 1.8 per cent; and γ globulin, 2.1 per cent.

The hematocrit of venous blood was determined in Wintrobe hematocrit tubes which were centrifuged for 30 minutes at 3,000 rpm. The blood urea nitrogen was determined by the method of Böger and Wezler (13).

RESULTS

The effects of *l*-norepinephrine and *l*-epinephrine on renal hemodynamics and urinary protein excretion are presented in Tables I and II and are illustrated in Figures 1 to 3.

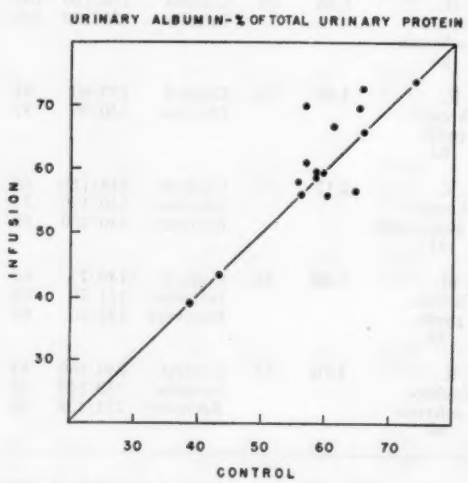

FIG. 2. URINARY ALBUMIN EXCRETION

Urinary albumin, expressed as percentage of the total urinary protein, during the infusion of *l*-norepinephrine and *l*-epinephrine is plotted against the control percentages. Only those subjects in whom the protein-inulin clearance ratio increased are included. The relative proportions of urinary albumin did not change during infusion of the adrenal medullary hormones.

TABLE II

Individual urinary and serum proteins during infusions of adrenal medullary hormones *

Subject	Procedure	Urine					Serum				
			Globulins					Globulins			
		Alb. %	α_1 %	α_2 %	β %	γ %	Alb. %	α_1 %	α_2 %	β %	γ %
					l-Norepinephrine						
R. H.	Control	65.6	7.4	6.0	9.6	11.4	50.0	5.6	16.1	13.6	14.6
	Infusion	72.6	5.6	6.5	9.1	6.2	49.8	5.8	16.0	13.7	14.6
	Recovery	74.8	6.0	4.8	8.4	6.0	51.0	5.1	16.0	13.9	14.9
T. P.	Control	61.0	10.1	8.7	9.9	10.3	29.9	5.7	17.9	21.1	25.5
	Infusion	66.8	9.5	7.0	8.7	7.9	33.8	5.9	17.9	17.5	25.0
A. K. (A)	Control	58.3	9.5	6.8	13.9	11.5					
	Infusion	59.6	8.8	7.5	13.8	10.3					
J. O'B.	Control	38.6	16.1	19.5	10.1	15.6	5.0	5.5	43.9	25.5	20.1
	Infusion	39.0	15.5	20.1	10.0	15.4	5.2	5.0	43.8	25.7	20.3
T. D.	Control	56.7	12.4	10.3	10.8	9.8	45.9	7.3	16.0	16.0	14.8
	Infusion	69.9	8.0	6.2	9.2	6.7	46.2	7.3	15.8	15.9	14.8
P. L. (A)	Control	65.8	10.6	6.3	10.2	7.0	37.2	7.3	12.8	16.8	25.9
	Infusion	65.8	10.9	6.8	9.9	6.5	37.8	7.3	12.4	16.6	25.9
	Recovery	62.8	9.8	6.9	10.0	10.5					
E. Z.	Control	56.0	14.3	9.5	12.2	8.0	29.9	8.2	35.5	12.1	14.3
	Infusion	56.0	13.3	9.6	13.2	7.9	28.3	8.0	37.0	12.0	14.6
W. E.	Control	65.0	8.4	8.4	12.0	5.8	40.1	10.0	21.0	16.7	12.3
	Infusion	69.6	7.6	7.6	10.5	4.7	43.1	10.7	18.5	16.1	11.7
	Recovery	64.0	8.6	9.7	11.8	5.9					
J. H.	Control	59.5	10.2	8.3	12.1	9.9	33.0	7.9	18.5	18.3	22.4
	Infusion	59.8	9.1	8.1	11.8	11.1	32.2	8.1	18.3	17.7	23.8
	Recovery	54.5	11.0	11.1	14.0	9.4	34.6	8.2	17.5	18.3	21.5
P. B.	Control	64.4	16.2†		8.9	10.5	52.4	6.5	12.3	15.6	13.2
	Infusion	56.8	17.8†		11.2	10.9	52.7	7.1	11.1	16.3	12.8
	Recovery	67.9	13.4†		10.3	8.5	52.5	6.0	12.0	15.1	13.4
					l-Epinephrine						
A. K. (B)	Control	56.8	8.7	6.2	14.5	13.8	39.3	6.1	12.1	17.7	24.7
	Infusion	61.0	7.3	6.8	14.2	10.7	38.0	6.6	11.0	17.9	26.4
P. L. (B)	Control	60.0	8.2	7.3	15.3	9.1	30.4	5.8	11.9	15.1	36.8
	Infusion	55.8	8.6	7.8	16.5	11.4	30.1	5.9	12.9	14.6	36.4
	Recovery	55.4	7.4	6.8	14.9	8.7	30.4	6.5	11.3	15.2	36.8
O. B.	Control	58.2	8.6	9.8	10.6	12.8	35.3	7.1	18.9	18.9	19.9
	Infusion	58.9	8.1	7.8	11.8	12.2	36.0	6.8	18.8	19.0	20.4
S. R.	Control	43.3	6.1	14.8	9.5	26.4	36.7	6.2	18.5	15.1	23.0
	Infusion	43.5	5.6	14.2	11.6	25.0	37.9	6.5	18.4	17.0	20.2
L. K.	Control	73.7	4.0	4.3	9.6	8.4	52.7	6.2	8.4	14.9	17.8
	Infusion	63.6	3.9	4.7	10.2	7.6	51.8	6.1	10.8	10.9	20.4
	Recovery	73.4	4.1	3.9	10.3	8.2	52.0	6.2	9.1	13.0	19.7
J. R.	Control	55.5	11.3	7.5	9.2	16.5	45.3	6.9	8.0	11.5	28.3
	Infusion	58.0	10.6	6.2	10.3	15.0	43.9	6.9	8.6	14.3	26.3
	Recovery	55.8	10.1	6.8	10.2	17.0	46.0	7.1	7.5	11.6	27.8

* All values were determined by paper electrophoresis and are expressed as percentages of the total urinary and serum proteins, respectively. *L*-norepinephrine or *l*-epinephrine was administered during the infusion period. The recovery periods were obtained immediately after cessation of the infusion of medullary hormones.

† α_1 plus α_2.

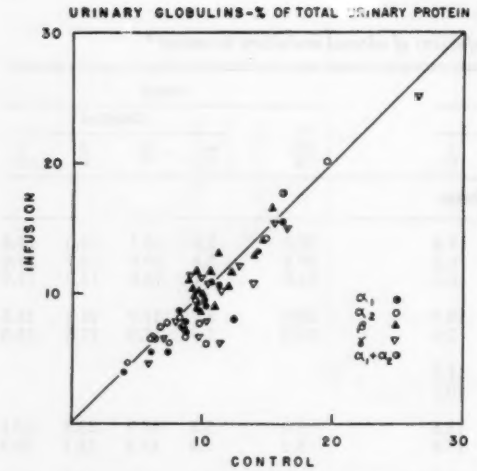

FIG. 3. URINARY GLOBULIN EXCRETION

The excretion of α_1, α_2, β, and γ globulin during the control periods, expressed as percentages of the total urinary protein, is plotted against the excretion of these fractions during the infusion of *l*-norepinephrine and *l*-epinephrine. Only those subjects in whom the protein-inulin clearance ratio increased are included. The relative proportions of the urinary globulins did not change during infusion of the pressor agents.

Systemic circulatory adjustments

There were no detectable changes in cardiac rate or arterial blood pressure during the administration of *l*-epinephrine. *L*-norepinephrine elicited adjustments similar to those reported previously in normal subjects (14). The systolic and diastolic blood pressures increased on the average 36 and 15 mm. Hg, respectively, and the cardiac rate diminished. On cessation of the infusion the blood pressure returned rapidly to control levels and the cardiac rate increased, often above control values.

Inulin and sodium p-aminohippurate (PAH) clearances

The averaged figures for inulin and PAH clearances during the control, infusion and recovery periods are tabulated in Table I and are arranged in a descending manner, according to the control inulin clearances. The changes elicited by *l*-norepinephrine and *l*-epinephrine were similar. The clearance of PAH diminished significantly in all but three subjects (E. B., J. B., and P. B.). On the average, PAH clearance decreased 23 per cent, with a range of 53 to 109 per cent of control values. The clearance of inulin remained constant in 12 subjects, decreased in 10 (12 to 61 per cent), and increased in 1 (E. B.). In subjects with severe renal functional impairment (W. E., J. H., O. B., and J. R.) the changes in the inulin clearance tended to be greater than in other subjects. The filtration fraction (C_{IN}/C_{PAH}) increased (10 per cent or more) in 16 subjects, decreased in 3 (J. B., O. B., and J. R.) and remained unchanged in four (E. B., J. H., J. M., and S. R.). During the recovery periods the clearances of inulin and PAH and the filtration fraction returned towards control values. PAH clearance exceeded control levels during recovery in 5 subjects (A. B., W. E., J. H., P. B., and J. R.). The clearance of inulin increased above control levels at this time in 3 subjects (A. B., W. C., and J. H.).

The similarity of these changes in renal clearances with those reported in normal subjects (14) suggests that the reduction in PAH clearance may be attributable, at least in part, to a reduced renal plasma flow. The associated reduction in the clearance of inulin and the rapid reversibility of the changes in PAH clearance support this view. Whether changes in the renal extraction of PAH also occurred was not established.

Urinary protein excretion

The effects of *l*-norepinephrine and *l*-epinephrine on urinary protein excretion ($U_{PR}V$, Table I) were similar. A variable response was observed. In 11 subjects (R. H., T. P., A. B., A. K.(A), J. O'B., T. D., P. L.(A), E. Z., W. E., A. K.(B), and P. L.(B)) the output of protein increased abruptly during the infusion of these agents. Protein output remained unchanged in 10 subjects and fell in 2 (O. B. and J. R.). Similar directional changes were observed in the clearance of protein, but since the concentration of serum proteins tended to rise, the magnitude of the alterations in protein clearance were less than those of protein excretion (C_{PR}, Table I). The mechanism of the rise in serum protein concentration is not clear but in view of the accompanying increment in the hematocrit of venous blood (Table I) this increase may be ascribed in part to hemoconcentration. A hemoconcentrating effect of epinephrine has been described previously (15).

A more consistent pattern of renal response was observed when the changes in protein clearance were related to changes in the inulin clearance. In 15 subjects the protein-inulin clearance ratio (C_{PR}/C_{IN}, Table I) increased during infusion of the adrenal medullary hormones (Figure 1). Thus, although protein excretion and clearance were unchanged in J. H. and S. R., and fell in O. B. and J. R., the protein-clearance ratio increased in all. In 8 subjects this ratio did not change. In three (J. B., E. B., and P. B.) of these subjects there were no associated adjustments of the renal circulation. Thus, in those instances in which renal vasoconstriction occurred, the protein-inulin clearance ratio increased in 15 and remained unchanged in 5. During the infusion the protein-inulin clearance ratio for the entire group of 23 subjects averaged 235 per cent of control values (range, 88 to 1146 per cent). This change was highly significant statistically ($t = 3.69$; $p < .01$). There was a significant correlation between the changes in this ratio and the changes in PAH clearance ($r = .565$; $p < .01$) (Figure 1). When the PAH clearance fell below 85 per cent of control values the clearance of protein increased relative to the inulin clearance (Figure 1). The changes in the protein-inulin clearance ratio could not be correlated with alterations in the inulin clearance or the filtration fraction since these parameters changed in a variable manner as protein output increased relative to filtration.

Following cessation of the infusion of the medullary hormones, the excretion and clearance of protein and the protein inulin-clearance ratio returned towards or to control values. In one subject (J. H.) protein excretion increased above the control level during recovery in association with renal hyperemia but the protein-inulin clearance ratio fell from the maximum value obtained during the pressor response to *l*-norepinephrine.

The relative proportions of the individual serum and urinary protein fractions did not change during the infusion of the medullary hormones or during recovery (Table II, Figures 2 and 3). In those subjects in whom the protein-inulin clearance ratio changed significantly during the infusion periods, there was no statistically significant change in the relative proportions of urinary albumin ($t = 1.93$; $p < .10 > .05$), α_1 globulin ($t = 1.17$, $p < .50 > .10$), α_2 globulin ($t = 2.53$; $p < .05 > .02$)[5], β globulin ($t = 1.21$; $p < .50 > .10$), or γ globulin ($t = 1.98$; $p < .10 > .05$).

DISCUSSION

The rate at which plasma proteins escape into glomerular filtrate in renal disease is governed by the characteristics of the protein molecules and by the structural and physiologic alterations in the glomerular membrane. The present study suggests that this process may be modified by the rate of blood flow through the kidney. When renal blood flow diminished as a result of the vasoconstrictive activity of the adrenal medullary hormones, *l*-norepinephrine and *l*-epinephrine, the rate of urinary excretion and renal clearance of plasma proteins increased abruptly. These alterations occurred independently of changes in systemic blood pressure and were not dependent upon but were influenced by changes in glomerular filtration rate. Thus, when the volume of filtrate diminished sharply as a result of intense renal vasoconstriction, as tended to occur in patients with severe renal functional impairment, less protein was available for excretion and the changes in protein output were not as apparent. However, the factors tending to accelerate the urinary loss of protein appeared to have been operative with equal force in these instances since the amount of protein excreted per unit of filtrate increased as it did when renal vasoconstriction was less intense and the changes in glomerular filtration rate were minimal (Figure 1).

These alterations in protein excretion may be attributed to either an increase in the transglomerular capillary transport of protein molecules or to a decrease in tubular reabsorption of protein, or to both. The present study did not distinguish between these possibilities. However, the similarity of the response in patients with renal disease with that observed in dogs in which the transglomerular transport of hemoglobin molecules increased during the pressor response to *l*-norepinephrine (10) suggests that the changes observed in the present study may be attributable, at least in part, to an accelerated transport of protein molecules across the glomerular membrane.

[5] This change was of borderline significance. In view of the small number of observations (14), statistical significance cannot be attached to this value.

Protein molecules apparently traverse capillary walls by both filtration and diffusion (2). The factors which govern the rates at which these processes occur are not entirely clear, but protein transport appears to depend, at least in part, upon the restricted area available for diffusion and upon a variety of physical factors which include the rate of hydrodynamic flow of fluid across the capillary wall and the thickness of the membrane through which flow occurs (2). According to the theory of molecular sieving the concentration of protein in capillary filtrates approaches that in the filtrand as the rate of transport of fluid across the membrane diminishes, owing to differences in the diffusion characteristics of protein and water molecules. This phenomenon has not been conclusively demonstrated in the kidney but has been suggested to account for the appearance of protein in the urine during circumstances in which momentary renal vasoconstriction occurs (2). However, this concept cannot account for the changes in protein output accompanying the renal vasoconstriction elicited in the present study by the adrenal medullary hormones since these changes occurred independently of alterations in glomerular filtration rate.

A more conventional explanation of the production or acceleration of proteinuria during renal vasoconstriction is that originally proposed in 1862 by Hermann (16) who suggested that these changes were brought about by an increase in capillary permeability secondary to anoxia. However, whether the dimensions of the channels through which the movement of protein molecules occurs are subject to variation under these and other experimental and physiologic conditions has not been clearly established. A change in pore size has been proposed to explain alterations in protein excretion not only during renal vasoconstriction but also under circumstances in which an increase in intraglomerular hydrostatic pressure was believed to have occurred, as during infusions of human serum albumin (3) and during the pressor response to renin (5).

Although these concepts must be considered in evaluating the changes in urinary protein excretion elicited by *l*-norepinephrine and *l*-epinephrine in the present study, certain evidence suggests that alterations in the size of the channels in the glomerular membrane secondary to either changes in intraglomerular hydrostatic pressure or to anoxia were not critical for the appearance of increased amounts of protein in the urine under the conditions of this study. Thus, protein output (or the protein-inulin clearance ratio) increased independently of changes in the renal filtration fraction, a parameter which may reflect corresponding adjustments of intraglomerular pressure provided the presence of vascular shunts within the kidney (17) does not invalidate interpretation of the filtration fraction in renal disease. Moreover, the observation that the clearances of the individual protein fractions increased equally in response to the medullary hormones (Figures 2 and 3) suggests that structural changes in the glomerular membrane secondary to an adjustment of glomerular capillary pressure or as a result of anoxia may not have been responsible since it appears unlikely that the transport of protein molecules of varying size and configuration would be equally affected by an increase in the dimensions of the channels through which this transport occurs. Furthermore, the rapid reversibility of the changes in protein output after cessation of the infusion of the medullary hormones tends to support the argument against structural alterations in the glomerular membrane, at least with respect to anoxia, since these alterations might have been expected to persist in part after the flow of blood through the kidney had been reestablished at or near its initial rate, thus prolonging the leakage of excess protein in the urine. This view would not apply, however, to an increase in the area available for diffusion brought about by adjustments of intraglomerular pressure since this change might be readily reversible as the hydrostatic pressure returned to control levels.

If these considerations suggest that the vasoconstrictive adjustments within the kidney did not affect protein output by altering the integrity of the glomerular membrane, they do not exclude the possibility that these adjustments altered protein output in some other manner. The correlation between protein clearance and the changes in PAH clearance (Figure 1) suggests that a reduction in the rate of blood flow through the kidney may have been responsible. The mechanism by which this circulatory adjustment may have acted is not clear. If under these conditions the rate and velocity of blood flow through the glomerulus diminished, the duration of contact between protein molecules and the capillary wall

may have been prolonged, thus allowing more time for the diffusion of these molecules into the glomerular filtrate. A similar hypothesis has been suggested to account for the increased excretion of hemoglobin in dogs during the pressor response to *l*-norepinephrine (10). Whether changes in the distribution of blood within the kidney (18) contributed to the results of the present study was not determined.

SUMMARY

A study was made of the effects of the adrenal medullary hormones, *l*-norepinephrine and *l*-epinephrine, on the urinary excretion and renal clearances of plasma proteins in 23 subjects with chronic renal disease and proteinuria. When these agents elicited moderate renal vasoconstriction the clearances of plasma protein increased abruptly. When the amount of filtrate was greatly reduced as a result of more intense vasoconstriction, the changes in protein excretion and clearance were less marked, although protein output increased relative to filtration, suggesting that the forces tending to accelerate protein excretion were operative in these instances as well.

These changes, which involved the individual protein fractions equally, occurred independently of alterations in systemic blood pressure, glomerular filtration rate and the renal filtration fraction, but were not observed in the absence of renal vasoconstriction and a reduction in renal plasma flow. The mechanisms responsible for altering protein output under these conditions were not determined. Although changes in tubular protein reabsorption and anoxic alteration of the glomerular membrane could not be excluded, it was suggested that the observed changes in protein output were attributable to an increased rate of diffusion of protein molecules into the glomerular filtrate owing to a more prolonged contact between these molecules and the capillary wall as a result of the slowing of blood flow through the glomerulus.

ACKNOWLEDGMENT

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THE EFFECT OF OBSTRUCTION TO BREATHING ON THE VENTILATORY RESPONSE TO CO_2 [1]

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In patients with pulmonary emphysema the increase in minute ventilation in response to the inhalation of CO_2 is much less than in normal individuals (1–4). This has been attributed to a decrease in the sensitivity of the respiratory center, perhaps the result of increased buffer base in chronic compensated respiratory acidosis. While the ventilatory response to CO_2 will obviously be limited by a restricted ventilatory capacity, most workers have felt that this defect could not account for the diminished response (1–5).

The present report is an attempt to evaluate the relationship between the ventilatory capacity and the response to CO_2 in normal subjects and patients with pulmonary emphysema. In normal subjects the ventilatory capacity was reduced by artificial airway obstruction, while in the emphysematous patients the ventilatory capacity was increased by the administration of a nebulized bronchodilator. The effect of these alterations in ventilatory capacity on the maximum ventilatory response to CO_2 and on the CO_2 stimulus-response curves was then determined.

METHODS

Six normal subjects, 5 males and 1 female, ranging in age between 20 and 35 and whose maximum breathing capacities ranged between 100 to 200 liters per min., and 24 emphysematous patients served as subjects. The diagnosis of emphysema was made clinically and was supported by spirographic evidence of a reduction of maximum breathing capacity and obstruction to expiratory outflow (Table I).

The vital capacity and maximum breathing capacity were measured on a 9 liter Collins Respirometer with valves and CO_2 absorber removed. The maximum breathing capacity was performed for 12 seconds, and the maximum of several estimations was taken and corrected to body temperature.

The maximum responses to carbon dioxide were measured with the same spirometer which was initially filled with 7 per cent CO_2 and 93 per cent oxygen. The subjects were instructed to expire maximally before commencing, in an attempt to reduce the effect of delayed mixing. They rebreathed the mixture to the end of their tolerance, more than five minutes in all cases, and the ventilation during the last minute was taken as their maximal response to CO_2.

All subjects were studied in the sitting position. The maximum breathing capacity and maximum ventilatory response to CO_2 were determined in emphysematous patients (subjects 1 to 20) at rest, and after the inhalation of nebulized bronchodilator (Vaponefrin®) in 15 of these subjects. The maximum breathing capacity and maximum ventilatory response to CO_2 were determined on separate occasions in the normal subjects at rest and while inspiring and expiring through three to seven increasing grades of artificial airway obstruction composed of cylinders containing porous bronze discs.

It is recognized that there is a large subjective component in this procedure. Alveolar gas samples were therefore obtained by the Haldane-Priestly technique during the resting states and at the termination of the procedure, and were analyzed in a Scholander Micro Gas Analyzer. The final alveolar CO_2 tensions were within 5 mm. Hg of each other in any one normal subject, indicating that there was a similar stimulus to respiration (pCO_2) while breathing through all resistances. In all subjects the final alveolar pCO_2 was between 65 to 80 mm. Hg. Also, the alveolar oxygen tension at the end of CO_2 breathing was over 200 mm. Hg, indicating that anoxia played no part in the respiratory response.

Ventilatory response curves were determined while breathing 3 per cent, 5.5 per cent and 7.8 per cent CO_2 in air. Subjects breathed each gas for 13 minutes and the ventilation was measured by collecting the expired gas in a Tissot spirometer for the last three minutes. Ventilation was expressed as the alveolar ventilation ratio as described by Gray (6). This is the ratio of the alveolar ventilation while breathing the various CO_2 mixtures, to the resting alveolar ventilation. Alveolar ventilation was calculated from the minute ventilation, respiratory rate, the dead space of the mouthpiece apparatus (50 cc.) and the dead space of the added resistance (50

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[2] Markle Scholar in Medical Science.

TABLE I

Ventilatory function in 24 male patients with pulmonary emphysema before and after bronchodilator

Subj. No.	Age yrs.	Ht. cm.	Duration of dyspnea yrs.	Vital capacity (cc.) Predicted	Vital capacity (cc.) Observed Before B.D.	Vital capacity (cc.) Observed After B.D.	Maximum breathing capacity (L./min.) Predicted	Maximum breathing capacity (L./min.) Observed Before B.D.	Maximum breathing capacity (L./min.) Observed After B.D.
1	59	174	10	3,800	1,390	1,700	98	17	23
2	57	172	8	3,670	1,475	2,150	92	16	22
3	64	164	2	3,220	3,300	3,140	82	62	69
4	49	179	13	3,980	3,300	3,140	109	61	78
5	41	178	11	4,090	4,150	4,100	116	85	98
6	27	177	1	4,300	4,650	4,610	129	79	90
7	31	177	1.5	4,200	1,640	2,780	124	76	97
8	71	167	15	3,250	1,360	1,690	79	20	20
9	26	169	2.5	4,000	4,200	5,000	123	102	138
10	59	178	4	3,890	4,140	4,560	101	103	134
11	52	174	16	3,710	4,160	4,540	101	67	84
12	74	181	13	3,640	3,600	4,000	86	86	109
13	35	189	10	4,550	5,630	6,100	130	86	130
14	71	177	2	3,580	2,500	4,360	86	32	69
15	55	184	12	4,050	2,410	1,600	107	46	59
16	71	162	2	3,050	1,860	2,110	75	40	54
17	39	171	3	3,900	1,400	1,430	113	39	42
18	62	160	10	3,100	865	1,250	81	10	16
19	59	178	12	3,800	2,460	2,380	99	26	34
20	40	175	11	4,000	4,690	4,710	114	131	169
21	57	171	8	3,550	1,900	2,950	94	20	32
22	74	167	10	3,150	2,815	3,370	75	26	37
23	71	177	2	3,500	2,500	4,400	86	31	69
24	71	161	2	3,000	1,800	2,110	75	40	54

cc.), assuming a dead space of 150 cc. in the normal subjects and 200 cc. in the emphysematous subjects. The range of anticipated error in the calculated alveolar ventilation ratio imposed by an error in estimated dead space volume of ± 50 cc. would be ± 0.15.

The CO_2 response curve was determined in four emphysematous patients (subjects 21 to 24) before and after the inhalation of the nebulized bronchodilator. Similar measurements were made on two normal subjects at rest and while breathing through a single resistance.

Normal subjects breathed through each resistance for five minutes before any measurements were made.

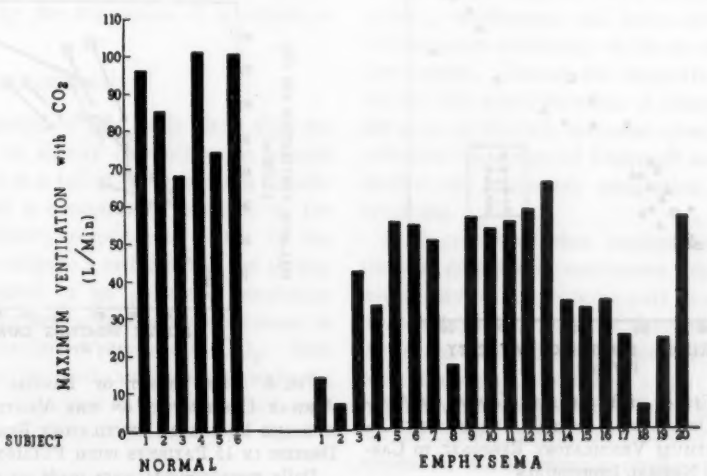

FIG. 1. THE MAXIMUM VENTILATORY RESPONSE TO CARBON DIOXIDE IN 6 NORMAL SUBJECTS AND 20 PATIENTS WITH PULMONARY EMPHYSEMA

RESULTS

Maximum ventilatory response to CO_2

The maximum ventilatory response to CO_2 in the six normal and 20 emphysematous subjects is shown in Figure 1. It can be seen that the emphysematous patients did not achieve as high a ventilation in response to the inhalation of CO_2 as did the normal subjects.

In Figure 2 the effects of increasing airway obstruction on the maximum breathing capacity and the maximum ventilatory response to CO_2 are shown in the six normal subjects. It can be seen that, as the maximum breathing capacity was reduced due to increasing airway resistance, the maximum ventilatory response to CO_2 was also reduced.

In Figure 3 the relationship between the maximum breathing capacity and the maximum ventilation with CO_2 in the 20 emphysematous patients is shown. It can be seen that this relationship in the emphysematous subjects was similar to that found when normal subjects breathed through an obstruction.

The effect of nebulized bronchodilators on the relationship between maximum breathing capacity and ventilatory response to CO_2 is shown in 15 patients in Figure 4. It can be seen that as the airway obstruction was reduced in each subject, as indicated by an increase in maximum breathing capacity, there was a concomitant increase in the maximum ventilation achieved with CO_2.

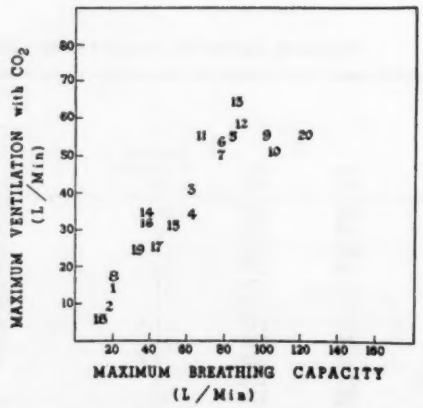

FIG. 3. THE RELATIONSHIP BETWEEN THE MAXIMUM VENTILATORY RESPONSE TO CARBON DIOXIDE AND THE VENTILATORY CAPACITY IN 20 PATIENTS WITH PULMONARY EMPHYSEMA

CO_2 stimulus-response curves

The CO_2 stimulus-response curves in two normal and four emphysematous subjects are shown in Figure 5. It can be seen that the mean slopes of the response curves are much lower than normal

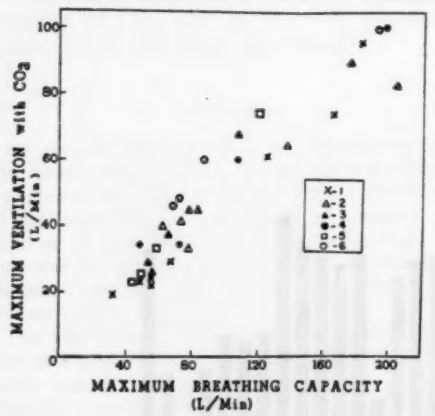

FIG. 2. THE EFFECT OF INCREASING GRADES OF ARTIFICIAL AIRWAY OBSTRUCTION ON THE VENTILATORY CAPACITY AND MAXIMUM VENTILATORY RESPONSE TO CARBON DIOXIDE IN 6 NORMAL INDIVIDUALS

Symbols are individuals and different points represent different resistances.

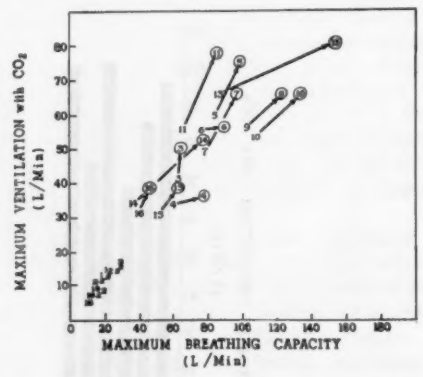

FIG. 4. THE EFFECT OF PARTIAL ALLEVIATION OF AIRWAY OBSTRUCTION ON THE VENTILATORY CAPACITY AND THE MAXIMUM VENTILATORY RESPONSE TO CARBON DIOXIDE IN 15 PATIENTS WITH PULMONARY EMPHYSEMA

Daily measurements were made on subjects 2 and 18 during intensive therapy directed at increasing the ventilatory capacity.

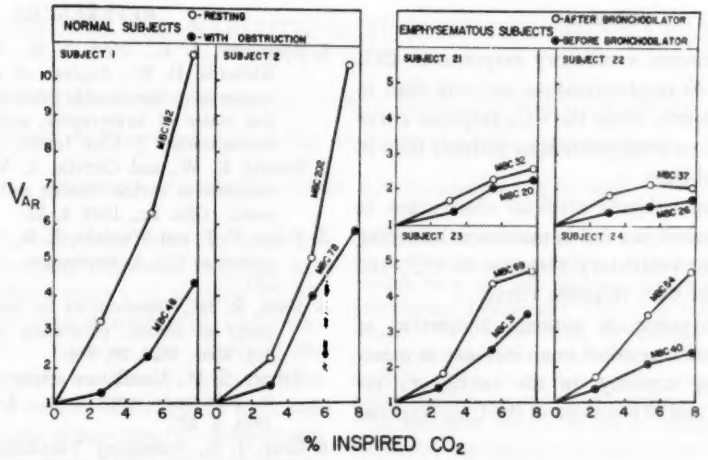

FIG. 5. THE CARBON DIOXIDE RESPONSE CURVES IN 2 NORMAL SUBJECTS AND 4 PATIENTS WITH PULMONARY EMPHYSEMA

Note the effect of increased airway obstruction in normal subjects and decreased airway obstruction in the abnormal subjects.

in the emphysematous subjects. It can also be seen that when normal subjects breathed through an obstruction, reducing the ventilatory capacity to the level of the emphysematous patients as indicated by the maximum breathing capacity, the mean slopes of the CO_2 response curves approached that of the emphysematous patients. In addition, it is noted that the mean slope of the response curve was increased in the four emphysematous subjects when the ventilatory capacity was increased by the inhalation of a nebulized bronchodilator.

DISCUSSION

The data reported in this study show that the introduction of an airway obstruction in normal subjects resulted in a fall in the maximum breathing capacity and a concomitant decrease in the maximum ventilatory response to CO_2. In the emphysematous subjects a reduction in the airway obstruction resulted in an increased maximum breathing capacity and a concomitant increase in the maximum ventilatory response to CO_2. This indicates that the reduced ventilatory capacity in itself may play a large part in the diminished ventilatory response to CO_2 in patients with pulmonary emphysema.

This conclusion is further substantiated by the results reported in regard to the CO_2 stimulus-response curves. These show that the mean slope of the response curve was markedly affected by the ventilatory capacity. When the ventilatory capacity of normal subjects was artificially lowered to the level of the emphysematous subjects, the stimulus-response curves obtained approached those of the emphysematous patients.

Tenney (5) and Alexander, West, Wood, and Richards (1) have also demonstrated a reduced CO_2 stimulus-response curve in patients with pulmonary emphysema and have attributed this to a diminished sensitivity of the medullary respiratory center. Tenney felt supported in this view because the administration of Diamox® increased the slope of the CO_2 response curve; but it is possible that the action of Diamox® was due to a reduction of pulmonary congestion and work of breathing.

It is concluded that emphysematous subjects show a diminished ventilatory response to CO_2 which may be due in large part to a reduced ventilatory capacity. This is presumably related to the increased work of breathing required to overcome bronchial obstruction (7). While the restricted ventilatory capacity may help to explain the diminished ventilatory response to CO_2, these results do not necessarily clarify the altered role of oxygen and carbon dioxide in the control of respiration in patients with pulmonary emphysema.

SUMMARY

1. The maximum ventilatory response to CO_2 was lower in 20 emphysematous subjects than in six normal subjects, while the CO_2 response curve was lower in four emphysematous patients than in two normal subjects.

2. In normal subjects artificial obstruction to respiration resulted in a fall in maximum breathing capacity, in the ventilatory response to CO_2 and in a diminished CO_2 response curve.

3. In emphysematous patients alleviation of airway obstruction resulted in an increase in maximum breathing capacity, in the ventilatory response to CO_2 and in the slope of the CO_2 response curve.

4. Little if any difference in the response to inhaled CO_2 was found between patients with obstructive disease and normal subjects with artificial airway obstruction.

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A STUDY ON THE NARCOTIC ACTION OF THE SHORT CHAIN FATTY ACIDS[1]

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That the short chain fatty acids have an inhibitory action on many metabolic reactions has been shown in baker's yeast (1), bacteria (2–4), fungi (5), cell-free yeast extracts (1), and mammalian muscle (6). However, the effects of the short chain fatty acids on "intact" animals have not been studied in similar detail, except for a few studies on the toxicity of butyrate (7), β-hydroxybutyrate (8, 9), acetone (10) and acetoacetate (11). The present work is a study of the narcotic action of the neutralized salts of the short chain fatty acids upon "intact" rats.

METHODS

Solutions of the fatty acid salts were prepared daily by adding a weighed amount of the acid (Fisher Scientific Co.) to distilled water and neutralizing it to a pH of 7.4 with 20 per cent w/v NaOH. The pH was determined with a glass electrode pH meter while air bubbled through the solution to insure adequate mixing. In this connection, pH determinations are subject to error because these acids tend to form two phase systems and colloidal gels. In the present experiments care was taken to neutralize all the free fatty acid and only homogeneous preparations were used. The concentration of the fatty acid anion is specified in the individual cases. The β-hydroxybutyrate was purchased as the sodium salt (Nutritional Biochemical Corp.) and solutions were prepared by dissolving a weighed amount in distilled water and bringing it to a pH of 7.4.

The rats used in these experiments were females from a Sprague-Dawley strain maintained on Nutrena® dog food nuggets and weighing between 50 and 200 grams. They were fasted 24 hours before experimentation and weighed immediately prior to the fatty acid injection. For species comparisons, mice, guinea pigs, dogs, chicks and frogs were used; however, detailed data were collected only with rats.

Nephrectomized rats were prepared according to the directions of Farris and Griffith (12). With the animal under ether anesthesia a dorsal midline incision was made, the renal connections isolated and tied off at the hilum, then the kidney excised. With the operation concluded, the incision was closed by two or three skin sutures and the area sealed with collodion. Rats were made alloxan-diabetic by a tail vein injection of alloxan monohydrate (Nutritional Biochemical Corp.), 40 mg. per Kg. body weight. Two weeks later, the animals were fasted 24 hours and then urine samples collected for glucose analysis. Only those rats with glucose in the urine greater than 1 per cent were considered alloxan-diabetic.

To determine whether or not an animal was conscious the animal was placed on its back at one minute intervals after the fatty acid solution was injected. If it did not right itself within ten seconds, it was judged unconscious. With most animals, and rats in particular, this gives a reproducible end point.

Urine analyses for glucose and acetone were carried out with commercially obtained tablets (Ames Co.). The determination of the fatty acids in the urine was done chromatographically (13).

RESULTS AND DISCUSSION

Intravenous or intraperitoneal injection of the sodium salts of the short chain fatty acids produces unconsciousness in rats, frogs, chicks, mice, dogs and guinea pigs. The effect of various amounts and concentrations of the short chain fatty acids given intraperitoneally in rats is presented in Table I. As can be seen, unconsciousness occurs in two to forty minutes after injection and may persist for as long as an hour. The amount of fatty acid anion which will produce unconsciousness in 50 per cent of a sample of rats (E_{50}) was determined by plotting the per cent of each group of animals that lost consciousness against the amount injected and then selecting the amount which corresponded to 50 per cent (14).

There is a definite relationship between the amount of the fatty acid which will produce unconsciousness and the carbon chain length of the compound. As might be expected, the E_{50} decreases rapidly with the increase in chain length (Figure 1).

It should be noted that the E_{50} for a given fatty acid depends upon the concentration of the acid as well as its chain length. This point is demonstrated in Figure 2 where the E_{50} for octanoate

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TABLE I

The effect of the intraperitoneal injection of the sodium salts of the short chain fatty acids

Rat no.	Fatty acid	Conc. *M*	Amount injected *mM/Kg.*	Response	Time until unconscious *minutes*	Duration unconscious *minutes*	E_{50} *mM/Kg.*
1	Acetate	1.0	20	None			
2			21	None			
3			30	None			
4			33	None			
5			36	None			
6		2.0	56	Unconsc.	18	15 (died)	
7	Propionate	1.0	28	None			
8			28	None			
9			28	None			
10			28	Unconsc.	18	18	
11			28	Unconsc.	20	36	
12			30	None			
13			30	None			29.0
14			30	Unconsc.	20	4	
15			30	Unconsc.	20	4	
16			30	Unconsc.	18	18	
17	Butyrate	1.0	12.5	None			
18			12.5	None			
19			12.5	None			
20			12.5	None			
21			12.5	None			
22			14.0	None			
23			14.0	None			
24			14.0	None			
25			14.0	Unconsc.	11	2	14.2
26			14.0	Unconsc.	11	3	
27			16.0	Unconsc.	14	6	
28			16.0	Unconsc.	8	20	
29			16.0	Unconsc.	8	21	
30			16.0	Unconsc.	7	11	
31			16.0	Unconsc.	7	20	
32			20.0	Unconsc.	11	5	
33			20.0	Unconsc.	9	51	
34			20.0	Unconsc.	8	40	
35			20.0	Unconsc.	7	50	
36			20.0	Unconsc.	6	42	
37		0.5	20.0	None			
38			20.0	None			
39			20.0	None			
40			20.0	None			
41			20.0	None			
42			23.0	None			
43			23.0	None			23.0
44			23.0	Unconsc.	24	2	
45			23.0	Unconsc.	14	12	
46			26.0	Unconsc.	14	1	
47			26.0	Unconsc.	12	5	
48			26.0	Unconsc.	12	7	
49			26.0	Unconsc.	11	9	
50	Valerate	0.67	18	None			
51			18	None			
52			18	Unconsc.	32	6	
53			18	Unconsc.	22	28	
54			18	Unconsc.	22	22	18.0
55			18	Unconsc.	22	26	
56			18	Unconsc.	22	30	
57			18	Unconsc.	21	44	
58			18	Unconsc.	20	28	
59		0.50	18	None			
60			18	None			
61			18	None			
62			18	None			

TABLE I—*Continued*

Rat no.	Fatty acid	Conc. *M*	Amount injected *mM/Kg.*	Response	Time until unconscious *minutes*	Duration unconscious *minutes*	E_{50} *mM/Kg.*
63	Valerate		18	Unconsc.	28	4	19.0
64			20	None			
65			20	None			
66			20	Unconsc.	38	10	
67			20	Unconsc.	18	30	
68			20	Unconsc.	16	16	
69			22	Unconsc.	24	8	
70			22	Unconsc.	16	8	
71			22	Unconsc.	14	44	
72			22	Unconsc.	12	32	
73	Caproate	0.50	12	None			
74			12	None			
75			12	None			
76			12	None			
77			12	Unconsc.	9	2	15.0
78			14	None			
79			14	None			
80			14	None			
81			14	None			
82			14	Unconsc.	8	6	
83			15	None			
84			15	None			
85			15	Unconsc.	9	5	
86			15	Unconsc.	8	4	
87			15	Unconsc.	6	6	
88			16	Unconsc.	9	7	
89			16	Unconsc.	7	7	
90			16	Unconsc.	6	10	
91			16	Unconsc.	5	9	
92			16	Unconsc.	4	10	
93	Heptanoate	0.50	5	None			
94			5	None			
95			5	None			
96			5	None			
97			5	None			
98			6	None			
99			6	None			
100			6	None			
101			6	Unconsc.	5	5	6.2
102			6	Unconsc.	5	6	
103			7	None			
104			7	Unconsc.	5	5	
105			7	Unconsc.	5	6	
106			7	Unconsc.	5	7	
107			7	Unconsc.	6	7	
108	Octanoate	1.0	2	None			
109			2	None			
110			2	Unconsc.	4	4	
111			2	Unconsc.	3	6	2.0
112			2	Unconsc.	2	10	
113		0.5	3.4	None			
114			3.4	None			
115			3.4	None			
116			3.4	None			
117			3.4	None			
118			3.6	None			
119			3.6	None			
120			3.6	None			3.7
121			3.6	Unconsc.	5	2	
122			3.6	Unconsc.	5	3	
123			3.8	None			
124			3.8	None			
125			3.8	Unconsc.	5	3	
126			3.8	Unconsc.	5	5	
127			3.8	Unconsc.	5	5	

TABLE I—*Continued*

Rat no.	Fatty acid	Conc. *M*	Amount injected *mM/Kg.*	Response	Time until unconscious *minutes*	Duration unconscious *minutes*	E_{50} *mM/Kg.*
128	Octanoate		5.0	Unconsc.	5	7	
129			7.5	Unconsc.	3	32	
130		0.1	6.0	Unconsc.	11	12	
131			6.0	Unconsc.	10	14	
132			6.0	Unconsc.	9	22	
133			6.0	Unconsc.	8	>32	
134			6.0	Unconsc.	7	>32	
135	Pelargonate	0.3	2.5	None			
136			2.5	None			
137			2.5	None			
138			2.5	None			
139			2.5	None			
140			2.7	None			
141			2.7	None			2.8
142			2.7	None			
143			2.7	None			
144			2.7	Unconsc.	8	2	
145			3.0	Unconsc.	5	4	
146			3.0	Unconsc.	4	8	
147			3.0	Unconsc.	3	6	
148			3.0	Unconsc.	3	10	
149			3.0	Unconsc.	3	10	
150	Caprate	0.1	2.8	None			
151			2.8	None			
152			2.8	None			
153			2.8	None			
154			2.8	None			
155			3.2	None			
156			3.2	None			
157			3.2	None			3.3
158			3.2	None			
159			3.2	Unconsc.	14	2	
160			3.4	None			
161			3.4	None			
162			3.4	Unconsc.	7	1	
163			3.4	Unconsc.	4	4	
164			3.4	Unconsc.	4	6	
165			3.6	Unconsc.	7	1	
166			3.6	Unconsc.	5	3	
167			3.6	Unconsc.	5	3	
168			3.6	Unconsc.	5	6	
169			3.6	Unconsc.	7	6	

as a function of concentration is presented. This pronounced effect of concentration upon the E_{50} is probably a result of a rapid disposal of the fatty acid anion and the influence of concentration upon the rate of absorption from the injection site. With propionate, solutions of low concentration are not effective enough to be adequately studied, whereas with pelargonate and caprate, only solutions of low concentration are sufficiently homogeneous to give reliable results. Therefore, the action of the three, nine and ten carbon members (propionate, pelargonate, and caprate respectively) cannot be quantitatively compared. However, their effectiveness corresponds with the general pattern as seen in Table I.

At the two carbon chain length the narcotic action becomes so weak that large quantities of acetate can be administered without the characteristic effect exhibited by the other acids. The injection of about 56 millimoles per Kg. body weight of 2 M sodium acetate will cause unconsciousness, but the pattern of the response is different. Following the injection of the other fatty acids there is reduced muscular tone and activity, whereas after the injection of the sodium acetate severe muscular spasms and convulsive movements occur. It is interesting to note that the intraperitoneal injection of the same amount (56 millimoles per Kg.) of 2 M NaCl produces the same type of response: that is, a few minutes after the injection the rat

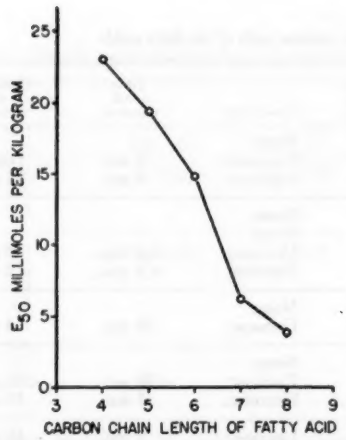

FIG. 1. E_{50} OF FATTY ACID ANION AS A FUNCTION OF CHAIN LENGTH

All acids neutralized to pH 7.4; conc. 0.5 M; given intraperitoneally. Data taken from Table I.

shows muscular twitching which becomes progressively increased until severe muscular spasms cause the animal to thrash about violently. The animal loses consciousness and dies, usually within an hour after the injection. The similarity of the response from the 2 M sodium acetate injection to that from the 2 M NaCl suggests that the mechanism of action here is related to the sodium ion rather than the acetate anion. This effect of high sodium ion has been reported by Ulrich and Shternov (15).

As might be expected, the length of time from injection until unconsciousness, the E_{50}, and the length of time of unconsciousness also depend to some extent upon the rate of absorption from the injected site. Consequently, subcutaneous administration is not very effective and nothing more than a sluggishness occurs. Further, the forced feeding of as much as twice the E_{50} will not even produce sluggishness. On the other hand, when the compounds are given intravenously unconsciousness occurs within a few seconds after the injection and with somewhat less than half of the amount necessary to cause unconsciousness with the intraperitoneal route (Table II).

Hemolysis

As it is common knowledge that washed red blood cells will hemolyze when suspended in a sufficiently concentrated solution of the fatty acid anions (16), it seemed reasonable to consider what part hemolysis might play in the reactions which follow fatty acid injection. Accordingly, we have drawn blood by cardiac puncture from rats made unconscious from a fatty acid injection and examined it grossly after centrifugation: in no instance was hemolysis noted.

Kidneys

The fatty acids and some of their metabolic products appear in the urine following their injection (Table III). From these findings we reasoned that the recovery from the narcotic action of the compounds rested mainly in excretion by the kidneys. But this idea was not supported by experiments on nephrectomized rats. The E_{50} and the length of time until recovery in nephrectomized rats seem to be of the same order of magnitude as in the control rats (compare Table IV with Table I).

Influence of (OH) group in carbon chain

To determine if the introduction of a hydroxyl group into the carbon chain would alter the action, we injected lactate and β-hydroxybutyrate and compared their effectiveness with the corresponding fatty acids, propionate and butyrate. The results show that the hydroxy compounds are much less effective (Table V): In fact, the E_{50} for 1 M lactate and β-hydroxybutyrate could not be de-

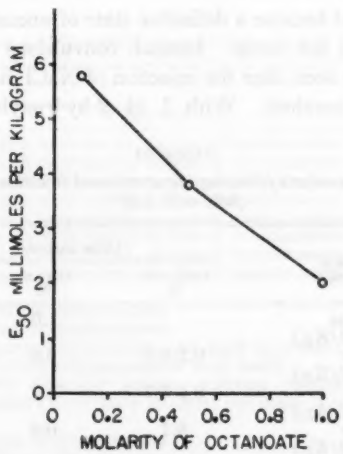

FIG. 2. E_{50} OF SODIUM OCTANOATE AS A FUNCTION OF CONCENTRATION

pH 7.4; given intraperitoneally. Data taken from Table I.

TABLE II

The effect of the injection site on the response to the sodium salts of the fatty acids

Rat no.	Injection site	Fatty acid	Conc. *M*	Amount injected *mM/Kg.*	Response	Time until unconsc.	Duration of unconsc.
170	Intravenous (tail vein)	Propionate	1.0	12.3	None		
171				18.0	Unconsc.	5 sec.	Died
172				19.0	Unconsc.	5 sec.	25 sec.
173		Valerate	1.0	2.0	None		
174				8.8	None		
175				10.0	Unconsc.	<1 min.	2 min.
176				12.0	Unconsc.	<1 min.	2 min.
177		Caproate	0.5	5.6	None		
178				6.2	Unconsc.	30 sec.	3 min.
179		Heptanoate	0.4	1.6	None		
180				2.0	Unconsc.	<30 sec.	<30 sec.
181				2.0	Unconsc.	5 sec.	55 sec.
182		Octanoate	0.5	0.8	Unconsc.	5 sec.	40 sec.
183				0.8	None		
184				1.0	Unconsc.	5 sec.	1 min.
185				1.2	Unconsc.	20 sec.	1 min.
186				1.5	Unconsc.	5 sec.	3 min.
187		Pelargonate	0.3	0.5	None		
188				0.5	None		
189				0.7	Unconsc.	5 sec.	
190				0.7	Unconsc.	5 sec.	160 sec.
191				0.7	Unconsc.	5 sec.	6 min.
192		Caprate	0.1	0.5	None		
193				0.6	Unconsc.	20 sec.	1 min.
194				0.8	Unconsc.	10 sec.	95 sec.
195	Subcutaneous	Valerate	1.0	22.0	None		
196	Gastro-intestinal (forced feeding)	Valerate	1.0	22.0	None		
197				22.0	None		
198				26.0	None		
199				35.0	None		

termined because a definitive state of unconsciousness did not occur. Instead, convulsions similar to those seen after the injection of NaCl discussed earlier resulted. With 1 M β-hydroxybutyrate these convulsions occurred after the administration of about 40 millimoles per Kg. body weight, whereas the E_{50} of 1 M butyrate is 14.2 millimoles per Kg. body weight.

TABLE III

Urine analysis following intraperitoneal injection of the fatty acid salts *

Fatty acid injected	Urine analysis		
	Fatty acid %	Glucose %	Ketones %
Propionate (30 *mM/Kg.*)		1.0	0.05
Butyrate (28 *mM/Kg.*)	0.2–0.3	0.0	2.0
Valerate (24 *mM/Kg.*)	0.4–0.7	1.0	0.0
Caproate (13 *mM/Kg.*)	0.2	0.0	2.0
Octanoate (3.9 *mM/Kg.*)	0.0 Octanoate 0.1 Butyrate		

* Rats fasted 24 hours before experiment. Urine collected for 6 to 12 hours after injection.

Alloxan-diabetic rats

Although the alloxan-diabetic rat does not develop ketosis as is seen in severe human diabetes (17), there is some disturbance of fat metabolism in alloxan diabetes (18) thus it seemed worthwhile to ascertain the susceptibility of alloxan-diabetic rats to the narcotic action of the fatty acid anions. The results of these experiments are given in Table VI. It is clear from these data that the alloxan-diabetic rats were actually less susceptible than normal rats.

Mechanism of action

The narcotic actions studied here are probably a direct action of the fatty acid anion on the cen-

TABLE IV

The effect of intraperitoneal injection of sodium butyrate and sodium valerate on nephrectomized rats

Rat no.	Fatty acid	Conc. *M*	Amount injected *mM/Kg.*	Response	Time until unconsc. *minutes*	Duration of unconsc. *minutes*
200	Butyrate	0.95	15.0	None		
201			15.0	None		
202			20.0	Unconsc.	11	10
203	Valerate	1.0	19.5	None		
204				None		
205				Unconsc.	28	4
206				Unconsc.	24	2
207				Unconsc.	24	30
208				Unconsc.	18	10
209				Unconsc.	18	22
210				Unconsc.	9	60

tral nervous tissue. The electroencephalographic changes which occur during the unconscious state support this idea (19). The fatty acid salts may inhibit the metabolic activity of cerebral tissue as they do muscle (6) and yeast (1). Several properties characterize their inhibition of yeast cell metabolism: 1. A nonspecificity, as evidenced by the large number of reactions affected, 2. An increasing action with an increase in chain length, 3. A reduction of the inhibitory action when a hydroxyl or carboxyl group is introduced into the hydrocarbon part of the molecules, 4. A reversibility.

The similarity of these properties with those demonstrated in the present study is certainly striking. However, this similarity does not necessarily mean that the cellular mechanism of action is the same in the two situations.

SUMMARY

Injection of the neutralized short chain fatty acids will produce unconsciousness in experimental animals. The amount of the fatty acid which will produce this response decreases with an increase in chain length. Further, the amount which will produce a loss of consciousness for a given fatty acid depends upon the concentration and the site of injection. The introduction of an (OH) group into the carbon chain reduces the narcotic action.

TABLE V

The effect of intraperitoneal injection of sodium lactate, sodium β-hydroxybutyrate and acetone on rats

Rat no.	Compound injected	Conc. *M*	Amount injected *mM/Kg.*	Reaction
211	Sodium	0.5	30	Sluggish movements, no loss of consciousness
212	lactate	1.0	30	Sluggish movements, no loss of consciousness
213			30	Sluggish movements, no loss of consciousness
214			34	Sluggish movements, no loss of consciousness
215			34	Sluggish movements, no loss of consciousness
216			36	Sluggish movements, no loss of consciousness
217			38	Sluggish movements, no loss of consciousness
218			38	Sluggish movements, no loss of consciousness
219	Sodium	0.5	30	No loss of consciousness
220	β-hydroxy	1.0	30	No loss of consciousness
221	butyrate		40	Severe muscular spasms and death in 57 min.; righting reflexes present until death.
222			50	Severe muscular spasms; righting reflexes present until death in about one hour.
223	Acetone	1.0	7	No loss of consciousness
224			37	No loss of consciousness
225			40	No loss of consciousness
226			50	No loss of consciousness
227			60	No loss of consciousness
228			80	Loss of consciousness for five minutes, approximately four minutes after injection.

TABLE VI

The effect of intraperitoneal injection of sodium valerate on alloxan-diabetic rats

Rat no.	Fatty acid	Conc. *M*	Amount injected *mM/Kg.*	Response	Time until unconsc. *minutes*	Duration of unconsc. *minutes*
229	Valerate	0.5	18	None		
230				None		
231				None		
232				None		
233			20	None		
234				None		
235				None		
236				Unconsc.	20	6
237			22	None		
238				None		
239				Unconsc.	28	4
240				Unconsc.	16	20
241			24	Unconsc.	38	2
242				Unconsc.	22	6
243				Unconsc.	16	12

There is no hemolysis from the amount of fatty acid which will produce unconsciousness. Nephrectomy does not seem to alter the duration of the narcotic action.

It is suggested that the mechanism of action at the cellular level may be the same as that in the fatty acid inhibition of yeast metabolism.

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THE EFFECT OF CHANGES IN BODY SODIUM ON EXTRACELLULAR FLUID VOLUME AND ALDOSTERONE AND SODIUM EXCRETION BY NORMAL AND EDEMATOUS MEN [1]

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The presence of a sodium-retaining hormone, aldosterone, in the adrenal gland and adrenal venous blood (1–4), the increased urinary excretion of aldosterone during sodium depletion (5, 6) and the inability of men and animals with adrenal insufficiency to conserve sodium support the concept that the process by which the sodium content and thus the extracellular fluid (ECF) volume of the body are regulated includes adrenocortical control of sodium excretion by aldosterone secretion (7).

That aldosterone is involved in the formation of edema is indicated by an increased urinary output of this hormone by patients with edema caused by heart failure, portal cirrhosis or nephrosis (8–13).

The present work further defines the role of aldosterone in the control of ECF volume of normal men and in the formation of edema by patients with heart failure or portal cirrhosis.

METHODS

The patients lived on a metabolic ward during the studies. Constant diets were prepared from uniform lots of food. The patients were weighed each morning after voiding and before breakfast. Blood samples were drawn 3 times a week before breakfast. Total collections of urine and feces were made. Urine was kept at 5° C. during and following collection. Sodium and potassium in diets, urine, feces and sera, and cation exchange resin in feces were determined as previously described (14). Uptake of sodium by resin was calculated on the assumption that there were 2 mEq. of sodium per day uncombined with resin in the stool (14).

$$\text{Uptake of sodium in per cent} = \frac{\text{mEq. fecal sodium/day} - 2}{\text{mEq. fecal resin/day}} \times 100.$$

Sodium uptake is thus the percentage of the total exchange capacity of the resin occupied by sodium. The uptake of potassium by resin was calculated in the same fashion on the assumption that there were 10 mEq. of potassium per day uncombined with resin in the stool. The results are essentially the same if it is assumed that all of the sodium and potassium in the stool is combined with resin.

Urinary extracts of aldosterone were prepared by the following procedure within three days of the collection of urine. The urine was acidified to pH 1 with HCl and divided into 1500 ml. batches. Water was added if necessary to achieve the proper volume. Each batch of urine was then placed in a glass tube 120 cm. high by 4.3 cm. in diameter. Urine was pumped from the bottom of this tube to the bottom of another tube 120 cm. high by 2.5 cm. in diameter. It ascended in droplets at the rate of 10 ml. per minute through 350 ml. of dichloromethane to the top of the tube whence it was returned to the top of the first tube. This extraction was continued for 22 hours. The dichloromethane had been previously purified by passage through a column of silica gel. Following the extraction the dichloromethane was washed twice with 0.1 its volume of 0.1 N sodium carbonate and twice with 0.1 its volume of water. The washings were backwashed with their volume of dichloromethane. The dichloromethane was then evaporated to dryness by passing nitrogen through it under reduced pressure at room temperature. The residue was dissolved in ethanol and stored at −7° C. until it was assayed in dogs for aldosterone (15). Urinary 17-hydroxycorticoids were determined in several patients by a modification of the method of Silber and Porter (16).

Venous pressure was measured by connecting a manometer directly to a needle inserted in an antecubital vein. With the patient reclining the venous pressure was recorded as the height of the fluid above a plane halfway between the uppermost portion of the sternum and the back of the patient.

PROCEDURE AND RESULTS

Dietary sodium for the different subjects varied from 6 to 12 mEq. per day. Dietary potassium was supplemented by potassium on cation exchange resin and in some cases by potassium salts.

[1] Parts of this work have been reported briefly in the discussion of the paper of Dr. John A. Luetscher, Jr. at the 1955 Laurentian Hormone Conference and in abstract form (Circulation, 1955, 12, 697).

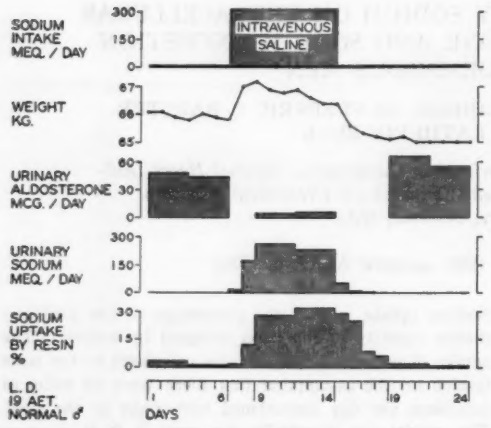

FIG. 1. STUDY OF A NORMAL MAN

During days 1 through 16 and days 19 through 24 he was depleted of sodium by cation exchange resin and a low sodium diet. During days 9 through 14 saline was given intravenously. Retention of enough sodium to produce a small expansion of extracellular fluid (ECF) volume as estimated from body weight led to a fall of urinary aldosterone to a very low level and to a greatly increased urinary excretion of sodium. During days 1 through 6 and days 19 through 24 the urinary sodium excretions were 0.6 and 0.7 mEq. per day.

Each subject ingested daily 355 mEq. (43 gm.) of a carboxylic cation exchange resin [2] in the hydrogen and potassium form. The intake of resin began some days before the collection of data. Whenever intravenous saline was given, the quantity given each day was 2 liters containing 253 millimols of sodium chloride and 55 millimols of sodium lactate. This was infused in a period of 2 hours or less. The excretion of 17-hydroxycorticoids, determined daily in several patients, was normal and did not vary during the studies.

[2] The preparation used was Natrinil, a pharmaceutical grade of Amberlite IRC-50 suitably prepared for therapeutic use.

Normal subjects

Two normal subjects were studied. The results of one study are shown in Figure 1. The results of the other study were essentially the same. Prior to the collection of data the subjects were depleted of sodium by resin and a low sodium diet. They were studied while depleted of sodium, then while receiving intravenous saline daily, and finally after being again depleted of sodium by resin and a low sodium diet. When each subject was depleted of sodium, his weight, urinary excretion of sodium, and sodium uptake by resin were lower and urinary excretion of aldosterone was higher than when body sodium was greater because of the daily intravenous infusion of saline. The average urinary excretion of aldosterone by each of the normal subjects during the days when intravenous saline was administered was 6 micrograms per day. Average serum sodium concentrations for the two subjects were slightly higher during the days that they received saline intravenously (Table I). During the days when saline was given intravenously, the uptake of potassium by resin was lower and the urinary potassium higher than during the days when the subjects were depleted of sodium. Serum potassium concentrations in both were slightly higher during the days that they received saline intravenously (Table I).

TABLE I

Serum electrolyte concentrations during sodium depletion and repletion*

Subject	Diagnosis	Serum sodium		Serum potassium	
		Sodium depleted	Sodium repleted	Sodium depleted	Sodium repleted
L. D.	Normal	139	141	3.9	4.1
W. Y.	Normal	139	140	4.4	4.5
F. H.	Portal cirrhosis	140	144	4.5	4.2
W. G.	Portal cirrhosis	140	145	3.0	3.6
J. L.	Arteriosclerotic heart disease	138	144	4.5	4.6
W. B.	Hypertensive heart disease	138	138	5.5	5.6
F. R.	Hypertensive heart disease	141	142	4.4	5.0
C. G.	Syphilitic aortic insufficiency	127	129	6.5	5.7
V. B.	Arteriosclerotic heart disease	142	128	4.3	5.6

* These values are the averages of the results of the determinations made during the periods of depletion and repletion.

Patients with cirrhosis

Two patients with portal cirrhosis were studied. Results from one study are shown in Figure 2. Those of the other study were essentially the same. During the first part of the study neither patient had subcutaneous edema. One had slight ascites; the other had no ascites. Saline was then given intravenously daily until subcutaneous edema and marked ascites were present. The weight of each patient increased 7 Kg. The daily infusion of saline was then discontinued, and the edema and ascites allowed to persist for a number of days. For one of the patients there was an additional period of study following removal of the ascitic fluid by paracentesis.

When edema and ascites were present, urinary excretion of sodium and sodium uptake by resin were higher and urinary excretion of aldosterone was lower than when there was no edema and little or no ascites. The urinary excretion of sodium and the uptake of sodium by resin never reached as high levels and the urinary excretion of aldosterone never reached as low a level as the values for the normal subjects when intravenous saline was given. The average amounts of aldosterone excreted in the urine by the two patients when

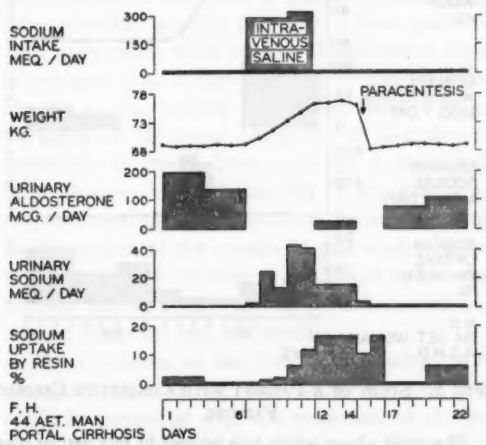

FIG. 2. STUDY OF A PATIENT WITH PORTAL CIRRHOSIS

During days 1 through 6 and days 17 through 22 there were slight ascites and no edema. Edema and conspicuous ascites were produced by the intravenous administration of saline. Associated with retention of enough sodium to produce this expansion of ECF volume there was a decrease of urinary excretion of aldosterone and an increase in the excretion of sodium.

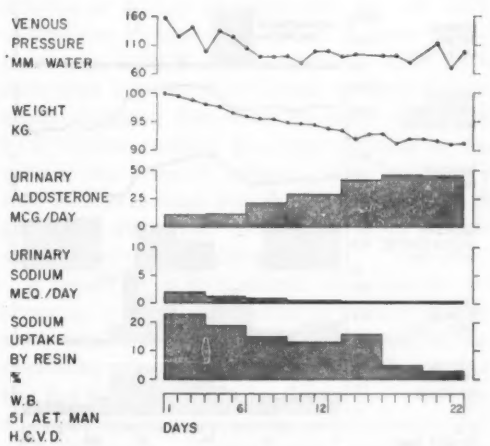

FIG. 3. STUDY OF A PATIENT WITH CONGESTIVE HEART FAILURE

The excretion of sodium on resin resulted in a gradual loss of edema which was not detectable after day 12. The loss of sodium and edema was accompanied by a marked decrease in the excretion of sodium on resin, a gradual decrease in urinary sodium excretion, and a marked increase in excretion of aldosterone.

edema was present were 12 and 26 micrograms per day. When edema was present the average concentrations of sodium in the serum were somewhat higher than when edema was absent (Table I). When edema and conspicuous ascites were present, potassium uptake by resin was lower and urinary potassium higher than when there was no edema and little or no ascites. When edema was present the average concentration of potassium in the serum was slightly higher in one case and slightly lower in the other case than when no edema was present (Table I).

Patients with congestive heart failure

Five patients with heart failure were studied. The diagnoses for these patients are given in Table I. Results of the studies of 3 patients are illustrated in Figures 3 through 5. The data from the studies of the other two patients were essentially the same as those illustrated in Figures 3 and 4.

The responses of 4 of the patients to changes in body sodium were qualitatively similar to the responses of the normal subjects and the patients with cirrhosis. J. L. who had been maintained free of edema by sodium restriction and diuretic

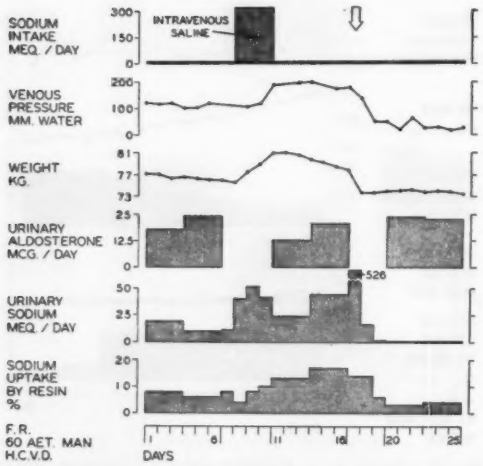

FIG. 4. STUDY OF A PATIENT WITH HEART FAILURE

The figure illustrates the small change in aldosterone excretion which may result from retention of enough sodium to produce a relatively large expansion of ECF volume. There was no edema on days 1 through 6 and on days 20 through 25. Edema was present on days 11 through 16. The arrow indicates the injection of a mercurial diuretic.

therapy was made edematous by the intravenous infusion of saline. He was studied as he lost edema as a result of a moderate urinary excretion of sodium and the fecal excretion of sodium on resin. W. B. (Figure 3) was studied as he passed from an edematous to a non-edematous state as a result of a large fecal excretion of sodium. F. R. (Figure 4) was studied while without edema, while edematous following the intravenous infusion of saline, and after loss of edema following use of a mercurial diuretic. C. G. was studied while only slight edema was present and after marked edema had been produced by intravenous saline. The changes in weight produced in these patients varied from 5 to 10 Kg.

When edema was present, urinary excretion of sodium and sodium uptake by resin were higher, and urinary excretion of aldosterone was lower than when edema was almost or completely absent. The urinary excretion of sodium and the uptake of sodium by resin never reached as high a level and the urinary excretion of aldosterone never reached as low a level as the values for the normal subjects during the days when saline was given intravenously. There was great variation in the responses of the patients with heart failure. In general, larger changes occurred in J. L. and W. B. than in F. R. and C. G. The average urinary aldosterone excretions of these patients while they were edematous were 13, 14, 17 and 306 micrograms per day. When edema was present the average serum sodium concentrations (Table I) were the same or slightly higher than when edema was absent. When edema was present, uptake of potassium by resin was lower and urinary potassium higher than when there was little or no edema. Serum potassium concentrations (Table I) were slightly higher in 3 cases when edema was present. It was somewhat lower in the fourth case. Venous pressure was much higher when edema was present.

The response (Figure 5) of the other patient (V. B.) with heart failure was opposite to that of the normal subjects. She had been in the hospital on a low sodium diet for a month prior to the study without evidence of change in cardiac function or in the gross edema that was present. During the first part of this study this gross edema

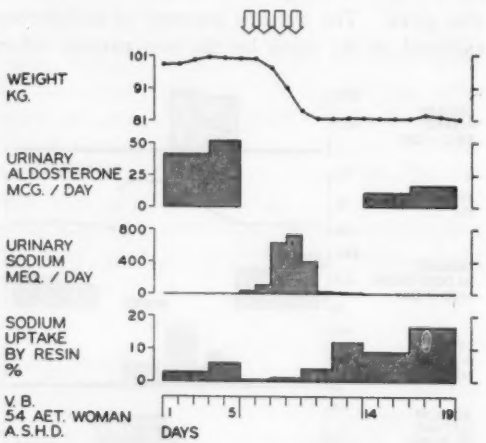

FIG. 5. STUDY OF A PATIENT WITH CONGESTIVE CARDIAC FAILURE

The great edema which was present in this patient was removed by sodium depletion with injections of a mercurial diuretic as indicated by the arrows. This was followed by a decrease in urinary excretion of aldosterone and an increase in the uptake of sodium by resin. The average urinary excretion of sodium during the first 5 days was 1.5 mEq. per day and during the last 6 days 2.6 mEq. per day. The response in this case was qualitatively opposite to that usually seen.

was allowed to persist. Then diuresis was produced by administration of a mercurial diuretic. She lost 20 Kg. Following this there was no subcutaneous edema. When edema was present, urinary excretion of sodium and uptake of sodium by resin were lower, and urinary excretion of aldosterone was higher than when there was no edema. Serum sodium concentration rose from 128 to 142 mEq. per liter as edema was lost.

Urinary potassium and uptake of potassium by resin were approximately the same when there was edema as when there was none. Serum potassium fell from 5.6 to 4.3 mEq. per liter as edema was lost. Loss of edema was accompanied by a reduction of venous pressure from 250 mm. to 150 mm. of water.

DISCUSSION

Variation in the excretion of an adrenocortical steroid may be due to changes in the plasma concentration of the steroid or to changes in renal function (17). That the variation in the urinary aldosterone observed in the present study was due to changes in plasma aldosterone is indicated by two facts. First, sodium uptake by resin varied inversely with urinary aldosterone excretion. Since intestinal excretion of sodium is reduced by adrenocortical steroids (18, 19), a low uptake of sodium by resin, when urinary aldosterone is high, is evidence that a high level of circulating aldosterone is also present. Secondly, there was no variation in the renal excretion of 17-hydroxycorticoids as would have occurred if there had been changes in renal function of importance in the excretion of steroids. Thus in the following interpretation of the results of this study plasma aldosterone concentration is considered to have increased and decreased as urinary aldosterone excretion increased and decreased.

The responses of the two normal subjects to changes in body sodium were studied so that they could be compared with the responses of the patients with cirrhosis or heart failure. When body sodium was maintained at a high level the normal subjects weighed about 1 Kg. more than when they were depleted of sodium. This increment in weight is taken as a moderately accurate measure of the increase in ECF volume produced by the increase in body sodium. Immediately after the infusion of 2 liters of saline each day the ECF volume was expanded by another 2 liters. Thus the change in ECF volume was at no time much more than 3 liters. The increase in body sodium which produced this relatively small change in ECF volume resulted in a large increase in the urinary excretion of sodium. The increase in body sodium in some way also led to a decrease of circulating aldosterone to very low levels as estimated from urinary aldosterone.

It appears that in a normal individual an increase in body sodium such as to produce a relatively small increase in ECF volume reduces the circulating aldosterone to very low levels and that this is a factor in increasing the urinary excretion of sodium and thus preventing continued expansion of ECF volume. Factors which were not measured in the present study may also, of course, have been of importance in producing the changes in sodium excretion which occurred. Glomerular filtration, for example, may increase with sodium loading of normal men (20–23). Thus an increase in glomerular filtration may have contributed to the increased excretion of sodium by the normal subjects.

The present data do not bear on the question of how an increase in body sodium modifies the level of circulating aldosterone.

The responses of the patients with cirrhosis and of 4 of the 5 patients with heart failure to an increase in body sodium were qualitatively normal but quantitatively weak in that urinary excretion of sodium did not increase to as high a level nor circulating aldosterone, as estimated from urinary aldosterone, decrease to as low a level as in the normal subjects who had much smaller increases in body sodium and ECF volume.

The failure of patients with cirrhosis or heart disease to reduce aldosterone to very low levels following an increase in body sodium is probably an important factor in the further retention of sodium and formation of edema by these patients. The other factor of known importance is glomerular filtration rate (24). That neither of these factors is alone sufficient to cause sodium retention is demonstrated by two observations. First, the reduction of glomerular filtration which occurs in adrenal insufficiency does not lead to sodium retention nor indeed stop sodium loss. Secondly, patients with tumors which produce large

amounts of aldosterone (25) do not retain much sodium.

It appears probable that one of the reasons that patients with cirrhosis do not respond quantitatively normally to an increase in body sodium and extracellular fluid volume is that most of the sodium and fluid is in the abdomen. The rest of the body may still have a low sodium and extracellular fluid content. The results of this study do not suggest any explanation of why patients with heart failure have a quantitatively weak response to retention of sodium and water.

The change in sodium excretion and aldosterone produced by retention of sodium in one patient was exactly opposite to the change produced in the other patients and normal subjects. That this is an unusual response is shown by its occurrence in only one of the patients in this study and in none of the patients in previous studies (14, 26) in whom the degree of edema was varied by sodium administration or deprivation, and sodium excretion in urine and onto resin determined. The occurrence of this unusual response indicates that some factor which did not play a decisive role in the other patients was decisive in this patient. One possibility is that this factor was the myocardial response to alteration in venous pressure. Reduction of the venous pressure of patients with heart failure may lead to improvement in cardiac function as measured by cardiac output (27). In this patient, retention of sodium and expansion of ECF volume may have increased venous pressure enough to impair myocardial function and thus have led to an increase in circulating aldosterone.

When the response of a patient with edema to further retention of sodium is qualitatively normal but weak, the accumulation of edema becomes progressively slower because of the increased sodium excretion that occurs as edema increases. However, despite a degree of edema that caused severe symptoms, the patients observed in this study could have maintained a steady state as far as ECF volume was concerned only if their dietary sodium were low. A patient who responds to an increase in body sodium with an increase in aldosterone is in a peculiarly unfavorable position. As edema accumulates he becomes even more prone to accumulate additional edema.

Potassium excretion on resin by patients and normal subjects was greater when body sodium and thus ECF volume were relatively low than when they were large. This may have been due in part to a direct effect of aldosterone on the intestinal excretion of potassium. However, it may have been due only to the presence of less sodium on resin when body sodium was low since a decrease in sodium on resin leaves more exchange capacity available for potassium.

The increased fecal excretion of potassium when body sodium was relatively low resulted in decreased net intestinal absorption of potassium. This led to decreased urinary excretion of potassium. This decrease in urinary potassium excretion was not due to a change in circulating aldosterone. It occurred despite an increase in circulating aldosterone which tends to increase potassium excretion (7).

SUMMARY

The urinary excretion of aldosterone and sodium and the fecal excretion of sodium onto cation exchange resin have been studied in normal men and in patients with heart failure or portal cirrhosis in whom the body content of sodium has been experimentally varied. Reasons are advanced for considering urinary aldosterone excretion a measure of circulating aldosterone.

A relatively small increase in the body sodium of normal men decreases the urinary excretion of aldosterone greatly. The decrease in circulating aldosterone which this reflects is probably an important factor in the increased urinary excretion of sodium which occurs at the same time. This response tends to prevent a continued increase in body sodium and thus extracellular fluid (ECF) volume.

Retention of enough sodium to produce marked expansion of the ECF volume of patients with heart failure or portal cirrhosis usually produces changes in aldosterone and sodium excretion qualitatively similar to those occurring in normal subjects with relatively small increases in body sodium and ECF volume. However, aldosterone excretion does not fall to as low a level nor sodium excretion rise as high as in normal subjects.

The failure of aldosterone to fall to very low levels in response to an increase in body sodium is probably an important factor in the formation

of edema in patients with heart failure or portal cirrhosis.

Addendum

Since the completion of this study, an abstract has been published stating that increasing dietary sodium decreases but does not return to normal the urinary excretion of aldosterone by patients with heart failure. (E. S. Gordon, J. Lab. & Clin. Med., 1955, **46**, 820.)

ACKNOWLEDGMENT

The dietetic aid of Miss Merme Bonnell is very gratefully acknowledged.

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THE REGULATION OF ALDOSTERONE SECRETION IN MAN: THE ROLE OF FLUID VOLUME [1]

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Although aldosterone has recently been shown to play a part in the retention of sodium by edematous subjects (5), the factors governing its production have not been defined.

It has been reported (6) that sodium restriction in normal subjects leads to increases in urinary aldosterone-like steroids, and that the quantities of such steroids are, other things being equal, inversely proportional to the amount of sodium excreted in the urine (5). In the studies reported here we have confirmed these reports in part, and have sought to clarify the mechanism or mechanisms by which changes in sodium balance lead to changes in aldosterone secretion. The evidence supports the view that some function of extracellular fluid volume, and not sodium *per se*, mediates the regulation of aldosterone secretion by dietary sodium.

METHODS

Studies were done on 18 normal control subjects, on one patient with diabetes insipidus and on one with panhypopituitarism. They were maintained on metabolic regimen, in air-conditioned environment; intake of food and fluids was changed only as the design of experiment required changes of salt and water intake. In four instances, only water, dextrose and sodium chloride were given throughout the study. Body weight was determined daily under standard conditions, and blood drawn at the start of metabolic days. Urine was collected in 24-hour lots. Serum and urine were analyzed for sodium and potassium by flame photometer, with lithium as internal standard, for chloride by the method of Van Slyke and Hiller (7) and for osmolality by the instrument designed by Bowman, Trantham, and Caulfield (8) using as standards sodium chloride solutions corrected for "true" osmolality with the use of the International Critical Tables (9). Urinary aldosterone-like steroids were estimated by bioassay in adrenalectomized dogs by a method previously described (10). For reasons defined in that paper, substances so determined are hereinafter referred to as aldosterone.

[1] These studies have been reported in part and summarized elsewhere (1–4).

RESULTS

I. The effect of sodium deprivation (Figure 1)

When dietary sodium was restricted to 10 or less milliequivalents a day there ensued in all cases 1) a loss of weight, 2) an increase of urinary aldosterone and 3) a decrease in urinary sodium excretion. When mercurial diuretics were given as sodium restriction was begun, these changes were brought about within one 24-hour period. They occurred in a patient with panhypopituitarism, and in four normal subjects whose intake consisted of water, glucose and sodium chloride alone. Similar results have been obtained by Luetscher and Axelrad (6) following sodium deprivation and mercurial diuretics and by Beck, Dyrenfurth, Giroud, and Venning following the sodium diuresis produced by Pitressin® and water (11). When no further procedure was carried out (see below) the increased excretion of aldosterone persisted until sodium was restored to the diet.

In the sodium-depleted subject, replacement of salt and water resulted in a prompt reversal of the changes described above, that is, in 1) weight gain, 2) a decrease of urinary aldosterone and 3) an increase in urinary sodium excretion. These changes are illustrated in Figure 1. The serum sodium concentration did not change significantly with deprivation or with restoration of sodium. Thus, water was lost and regained *pari passu* with sodium.

II. The effect of expansion of body fluids

In order to separate the role of sodium from that of water in the above phenomena, four subjects were depleted of sodium and maintained on

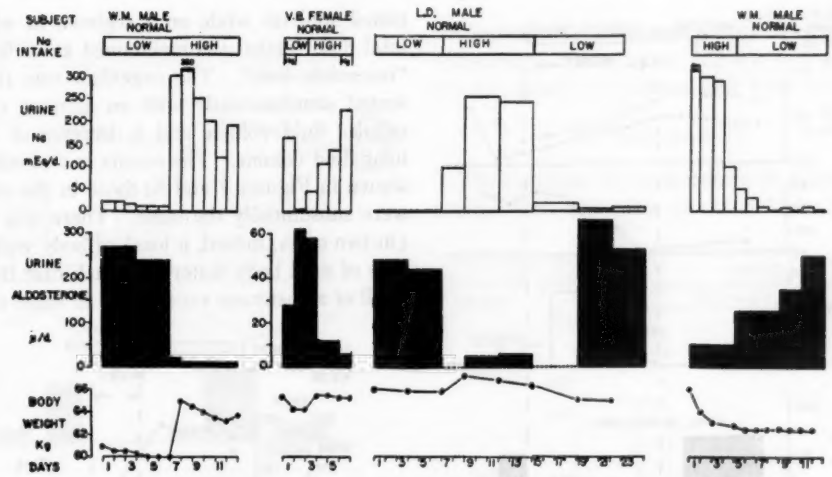

FIG. 1. SODIUM INTAKE, URINARY SODIUM AND ALDOSTERONE, AND BODY WEIGHT IN NORMAL SUBJECTS

Hg indicates mercurial diuretics. Note that in V. B. a mercurial given together with sodium did not affect aldosterone excretion; this was demonstrated in 2 other subjects.

In this and subsequent charts, urinary aldosterone is represented in solid black. When the zero line alone is shown, assays were not done. μ indicates micrograms.

constant, low sodium diets, the water content of which was estimated to approximate the "insensible loss" of water. Pitressin® Tannate in Oil was administered to maintain antidiuresis, and additional water was then administered by mouth. The organism was thus presented simultaneously with an increase of fluid volume and a decrease of serum sodium concentration and osmolality. The results in two subjects are shown in Figures 2 and 3. Those in the other two subjects were substantially the same. Expansion of body fluids with water resulted in gain of weight, hyponatremia, and decreases in urinary aldosterone. In each case there was an increase in urinary sodium excretion and a decrease in urinary potassium excretion—physiological changes one would expect following decreases in aldosterone secretion (10).

When Pitressin® was stopped and water loss permitted, aldosterone excretion rose in all cases as urinary sodium excretion fell and urinary potassium excretion rose.

III. The effect of contraction of body fluids

If the water loading produced decreases in aldosterone secretion in these subjects through its contribution to body fluid volume, it follows that contraction of body fluids, without sodium deprivation, should lead to a rise in aldosterone secretion. To test this hypothesis, a patient with diabetes insipidus, one with panhypopituitarism, and 5 normal subjects were subjected to dehydration while receiving diets containing moderate amounts of sodium. The organism was thus simultaneously presented with a decrease of fluid volume and an increase of serum sodium concentration and osmolality. Some of the results are shown in Figures 4, 5, 6. In the patient with diabetes insipidus, (Figure 4), Pitressin® Tannate in Oil was administered at constant dosage during control periods. During experimental periods, (Figure 4, days 4 and 13) the Pitressin® was discontinued for 36 hours. There ensued diuresis, loss of weight, hypernatremia and increases in aldosterone excretion. In both instances there was a decrease in urinary sodium excretion and an increase in urinary potassium excretion—physiological changes one would expect following increases in aldosterone secretion (10).

In the other subjects (Figures 5 and 6), the changes were similar, although the degree of dehydration and the resulting rise in aldosterone excretion were quantitatively much less.

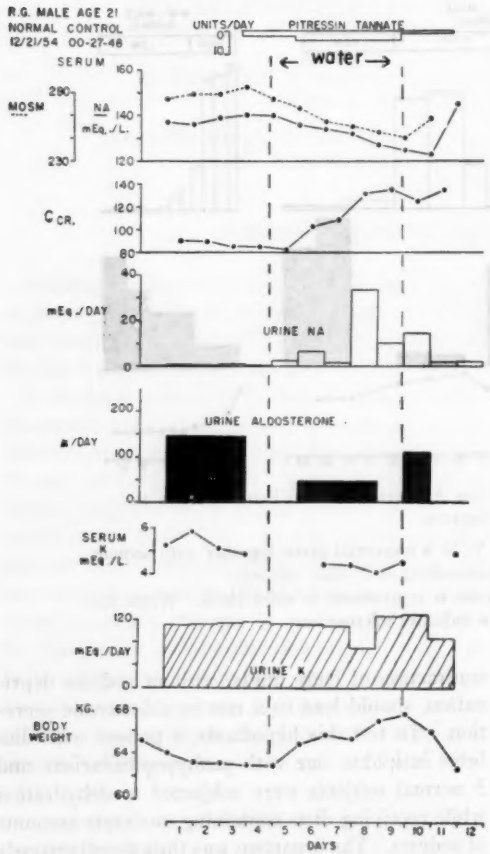

FIG. 2. SERUM SODIUM, OSMOLALITY AND POTASSIUM, CREATININE CLEARANCE, URINE SODIUM, ALDOSTERONE AND POTASSIUM, AND BODY WEIGHT IN A NORMAL, SODIUM-DEPLETED SUBJECT ON LOW AND HIGH WATER INTAKES

One to three liters of water were added to the intake on days 5 through 9. Sodium intake was 9 mEq. a day throughout.

IV. The effect of expansion of extracellular fluid volume and simultaneous contraction of intracellular fluid volume

In view of the above observations, and of the reciprocal relationship between aldosterone secretion and the urinary excretion of sodium, chief cation of the extracellular fluid, it is reasonable to suppose that it is the extracellular fluid rather than total body fluid whose volume influences aldosterone secretion. This was tested directly by administering hypertonic saline to four sodium-depleted subjects while on a regimen in which the total fluid intake was maintained at or below the "insensible loss." The organism was thus presented simultaneously with an increase of extracellular fluid volume and a decrease of intracellular fluid volume. The results in two studies are shown in Figures 7 and 8; those in the other two were substantially the same. There was no gain (in two cases, indeed, a loss) of body weight, and thus of total body water. In each case there was a fall of aldosterone excretion. In three cases the

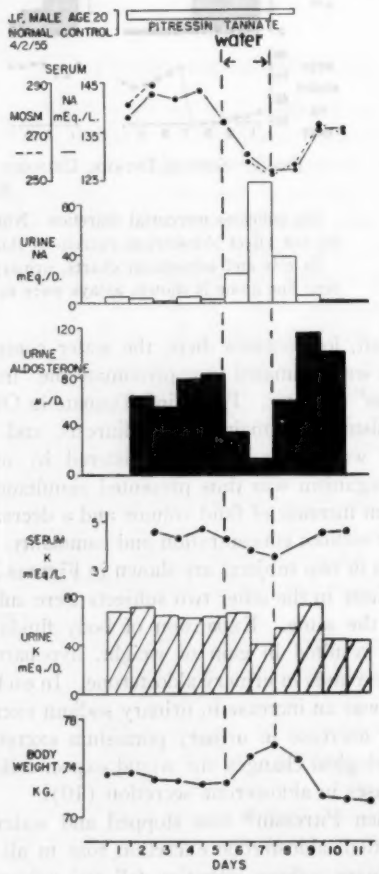

FIG. 3. SERUM SODIUM, OSMOLALITY AND POTASSIUM, URINE SODIUM, ALDOSTERONE AND POTASSIUM, AND BODY WEIGHT IN A NORMAL SODIUM-DEPLETED SUBJECT ON LOW AND HIGH WATER INTAKES

Four liters of water were added to the intake on day 6, and 2 liters on day 7. Data on day 5 were "corrected" for loss of urine with creatinine data. Sodium intake was 10 mEq. a day throughout.

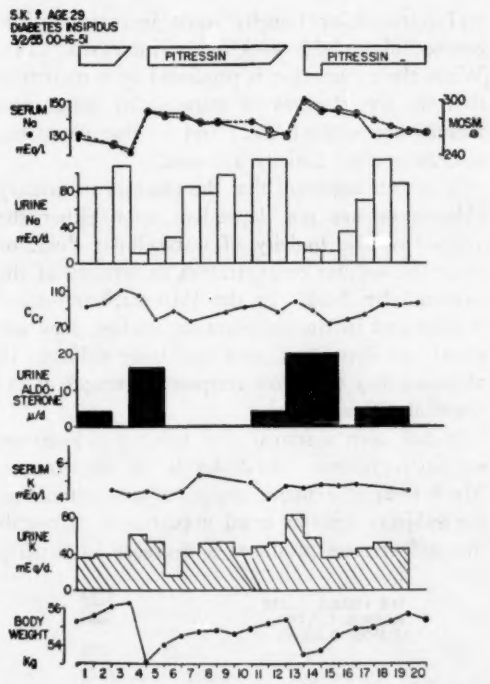

FIG. 4. SERUM SODIUM, OSMOLALITY AND POTASSIUM, CREATININE CLEARANCE, AND URINE SODIUM, ALDOSTERONE AND POTASSIUM IN A SUBJECT WITH DIABETES INSIPIDUS DEHYDRATED BY WITHDRAWAL OF PITRESSIN®

Sodium intake was 100 mEq. a day throughout.

magnitude of the expansion of extracellular volume was estimated from "chloride space" calculations. It was 1.7, 3, and 2.2 per cent of body weight.

V. Comparison of the effect of total body water loss with that of extracellular fluid loss in the same subject

In four subjects the effect of simple dehydration was compared, on an otherwise identical regimen, with that of predominantly "extracellular dehydration" (12) produced with mercurial diuretics. Such a comparison is shown in Figure 6. Table I shows a comparison of the total weight loss, the calculated extracellular fluid loss, and the rise in urinary aldosterone in 4 subjects in whom such direct comparisons were made. As noted above, the mercurial diuretics produced no changes in serum sodium concentration. It is apparent that changes in total body water were less effective in influencing aldosterone excretion than much smaller changes in extracellular fluid volume.

DISCUSSION

These studies show decreases of urinary aldosterone with expansion of extracellular fluid vol-

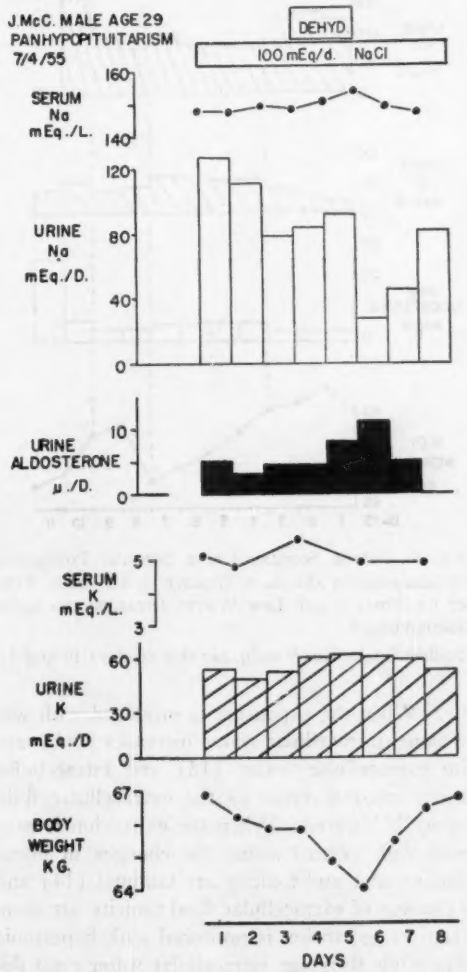

FIG. 5. SERUM SODIUM AND POTASSIUM, URINE SODIUM, ALDOSTERONE AND POTASSIUM, AND BODY WEIGHT IN A SUBJECT WITH PANHYPOPITUITARISM ON NORMAL AND LOW ("DEHYD.") WATER INTAKES

Sodium intake was 100 mEq. a day throughout.

A very high aldosterone excretion at the start of day 6 is indicated by the high "average" for this day despite starting hydration.

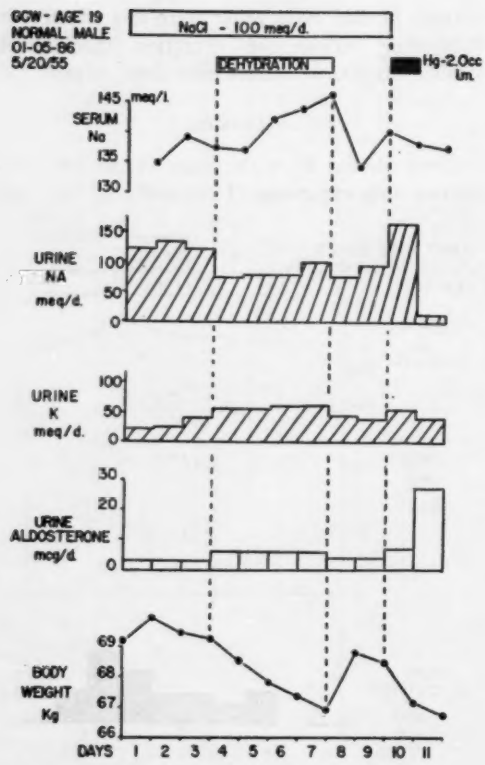

FIG. 6. SERUM SODIUM, URINE SODIUM, POTASSIUM AND ALDOSTERONE AND BODY WEIGHT IN A NORMAL SUBJECT ON NORMAL AND LOW WATER INTAKES AND AFTER MERCUHYDRIN®

Sodium intake was 9 mEq. per day on days 10 and 11.

ume. When the expansion is produced with water alone, intracellular water increases *pari passu* with extracellular water (13) and intracellular tonicity must decrease as the extracellular fluid tonicity is lowered. When the expansion is produced with normal saline the changes in intracellular water and tonicity are minimal (14) and no changes of extracellular fluid tonicity are seen. When the expansion is produced with hypertonic saline while thirsting, intracellular water must decrease, and intracellular tonicity increase, as water leaves the cells to enter the extracellular compartment.

Conversely, increases of urinary aldosterone were shown with contraction of extracellular fluid volume. When the contraction is produced by simple dehydration, intracellular water decreases and intracellular tonicity must increase, as the extracellular fluid tonicity is increased (14). When the contraction is produced by a mercurial diuretic, the changes in intracellular water and tonicity are minimal (12) and no changes of extracellular fluid tonicity are seen.

It is thus apparent that the changes in urinary aldosterone are not dependent upon either the volume or the tonicity of intracellular fluid, or upon the sodium concentration or tonicity of the extracellular fluid. In the Pitressin®-and-water studies and in the dehydration studies, they are clearly not dependent upon total body sodium. In all cases they do follow reciprocal changes in extracellular fluid volume.

It has been assumed that urinary aldosterone excretion reflects blood levels of aldosterone. Much indirect evidence supports this assumption for subjects without renal impairment. Crystalline aldosterone produces a decrease in urinary

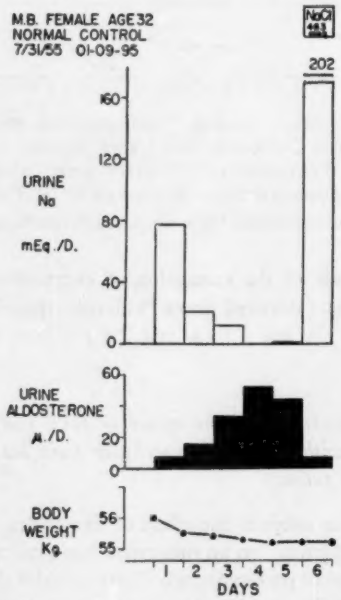

FIG. 7. URINARY SODIUM AND ALDOSTERONE, AND BODY WEIGHT IN A NORMAL SUBJECT WHO RECEIVED HYPERTONIC SALINE INTRAVENOUSLY ON RESTRICTED WATER INTAKE

Note that aldosterone fell with extracellular fluid expansion without gain in weight or total body water.

Oral sodium intake was 9 mEq. a day throughout. On day 4, 463 mEq. of 3.0 per cent saline were given intravenously.

sodium excretion and an increase in urinary potassium excretion (3, 10, 15). Thus the metabolic data should supply evidence as to whether the changes of urinary aldosterone reflect similar changes in serum aldosterone levels, and are not results of changes in renal clearance of the steroid. When body fluids were expanded with water (Figures 2 and 3), the decreases of urinary aldosterone were always accompanied by increases in urinary sodium and decreases in urinary potassium. When body fluids were contracted as a result of water loss alone (Figures 4, 5, and 6), the increases of urinary aldosterone were accompanied by decreases in urinary sodium; where the contraction was quantitatively greatest (Figure 4) there were marked increases in urinary potassium. This provides strong presumptive evidence that the urinary values do in fact reflect serum levels. In further support of this assumption is the fact that endogenous creatinine clearances indicated that increases in glomerular filtration rate accompanied the decreases in urinary aldosterone in the "expansion" studies (Figures 2 and 3) whereas decreases in filtration rate accompanied the increases in urinary aldosterone in the "contraction" studies (Figure 4).

In a previous study (16) it was shown that expansion of body fluids with water produced urinary loss of sodium, and it was suggested that this loss was in part a homeostatic response to the increase of body fluid volume. Increases of glomerular filtration rate accompanied the sodium loss, however, and a direct measure of aldosterone excretion was not then available. In that study, expansion of body fluid volume was accompanied by a fall in urinary potassium excretion during the period of expansion in all of the normal subjects but one—a finding in accord with the view that there were indeed decreases in circulating aldosterone during expansion.

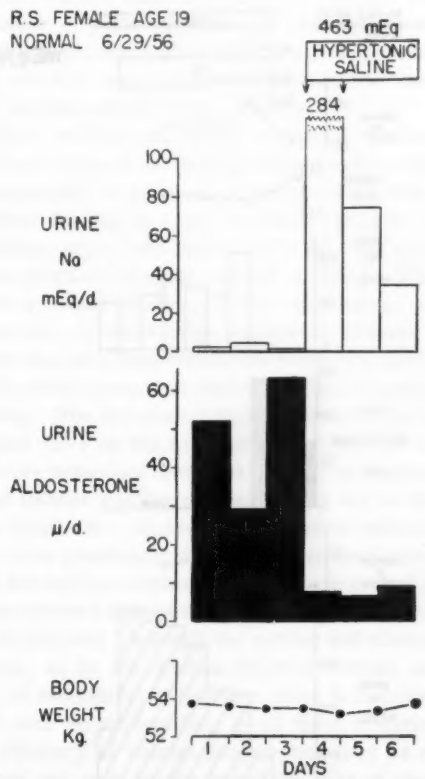

FIG. 8. URINARY SODIUM AND ALDOSTERONE AND BODY WEIGHT IN A NORMAL SUBJECT WHO RECEIVED HYPERTONIC SALINE INTRAVENOUSLY ON RESTRICTED WATER INTAKE

Note that aldosterone fell with extracellular expansion without gain in weight or total body water. Oral sodium intake was 9 mEq. a day throughout.

It is assumed that changes in circulating aldosterone reflect changes in aldosterone secretion,

TABLE I

Comparison of the effect, in the same individual on the same regimen, of simple dehydration with that of "extracellular dehydration" on aldosterone excretion

	Dehydration					"ECF dehydration"				
			"Chloride space" loss	Aldosterone μgm./day				"Chloride space" loss	Aldosterone μgm./day	
Subject	Wt. loss Kg.	% B.W.	L.	Initial	Final	Wt. loss Kg.	% B.W.	L.	Initial	Final
J. McC.	1.79	2.7	0.47	4.5	11	0.32	0.5	1.15	3	19
V. B.	1.32	2.0	0.50	12	21	1.13	1.7	1.81	27	62
S. Z.	1.93	3.0	0.36	3	9	1.00	1.6	1.53	9	17
G. W.	2.29	3.3	0	3	6	1.73	2.5	2.06	4	26

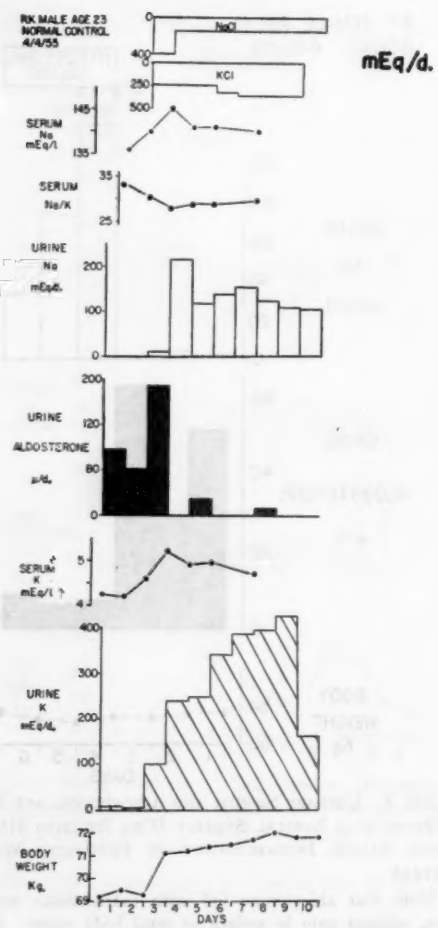

FIG. 9. SERUM SODIUM, POTASSIUM AND SODIUM-TO-POTASSIUM RATIO AND URINARY SODIUM, ALDOSTERONE AND POTASSIUM IN A NORMAL SUBJECT ON BALANCE REGIMEN UPON LOADING WITH POTASSIUM AND WITH SODIUM AND WATER SIMULTANEOUSLY

A single mercurial diuretic had previously been given to produce sodium depletion, and an ion exchange resin fed for one day to produce potassium depletion. Note the fall in aldosterone excretion despite the rise in serum potassium and the fall in serum sodium-to-potassium ratio. The data suggest substantial rise in total body potassium during "loading."

although the possibility that they reflect reciprocal changes in its destruction cannot be rigidly excluded.

It has been shown that potassium loading may produce increases, and potassium depletion decreases, in aldosterone secretion (1, 2, 17), even when such procedures do not result in reciprocal changes in sodium balance (2). The mechanism by which potassium exerts these effects is obscure. The changes in aldosterone secretion described in the present study could be shown to occur without significant changes in a) serum potassium, b) the ratio of serum sodium to serum potassium, or c) total body potassium. In four cases, rises of aldosterone secretion were produced by mercurials in the absence of dietary potassium, in contrast to the findings of Laragh and Stoerk (17). In the study shown in Figure 9, very large increases in serum potassium and presumably in total body potassium, and a striking decrease in the serum "sodium-to-potassium ratio" were produced with potassium loading, but a concomitant rise in extracellular fluid volume was induced, and aldosterone excretion fell. Similar results were obtained in two other subjects. Thus the effects of fluid volume changes on aldosterone secretion are not mediated by any demonstrable changes in potassium metabolism.

The present study shows changes in aldosterone secretion which paralleled, and thus did not tend to oppose, changes in serum sodium concentration —that is, decreases in aldosterone secretion could be induced in spite of hyponatremia and increases in spite of hypernatremia. The question arises as to whether changes in serum sodium concentration, *per se*, ever give rise to compensatory changes in aldosterone secretion. Our studies give little support to such a mechanism. (Indeed, when effects on renal hemodynamics are excluded there appears to be no evidence [18] that sodium conservation can be induced by hyponatremia, or sodium loss by hypernatremia.) This adrenal cortical independence of concentration is, of course, in contrast to the dependence (19) of the supraoptico-hypophyseal system on effective serum osmolality. We suggest that the normal control of sodium and water homeostasis depends on a dual "feed-back" mechanism, as illustrated in Figure 10, where aldosterone is shown to produce an increase in serum sodium concentration, which results (through decrease in water excretion mediated by antidiuretic hormone and/or through increase in water intake) in a rise of extracellular fluid volume. Some function of the volume increase then serves to inhibit aldosterone secretion. We have no evidence relating to the mechanism

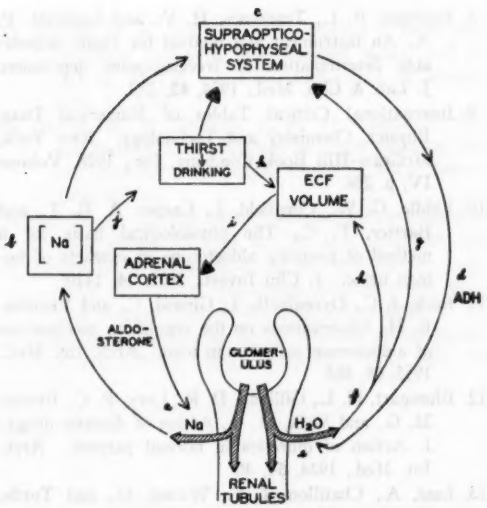

FIG. 10. A SIMPLIFIED SCHEMA TO REPRESENT NORMAL CONTROL OF SODIUM AND WATER HOMEOSTASIS

Aldosterone increases tubular sodium transport (a) thus tending to increase serum solute concentration (b). This stimulates the supraoptico-hypophyseal system (c) to produce antidiuretic hormone (ADH) (d) which promotes water reabsorption (e). The water so reabsorbed (f) lowers the serum solute concentration (g), thus inhibiting further ADH output, but also contributes to ECF volume (h). Some function of ECF volume tends to inhibit further aldosterone output (i).

Alternatively, increase of serum solute concentration (b) may lead to thirst (j) and thus directly to an increase in ECF volume (k). The sodium intake is assumed to be constant.

The proposed schema omits reference to the effects of volume changes *per se* on ADH secretion (20, 21) as not germane to the present material. It also ignores renal hemodynamic changes.

→ = stimulation
↛ = inhibition

by which effects of volume changes may be transmitted to the adrenal cortex.

It has been shown (5, 22–26) that nephrosis, cirrhosis with ascites, and cardiac failure are characterized by aldosterone secretion in excess of normal in spite of increases in extracellular fluid volume. The present studies do not, of course, explain the pathological physiology of aldosterone secretion in these diseases. It has been found (22), however, that even when these conditions are present, some function of extracellular fluid volume can still exert some control over the amount of aldosterone secreted. If the "effective" volume resides within the vascular compartment (3) the stimulus to aldosterone secretion in nephrosis and cirrhosis with ascites may be a result of loss of such volume into the tissues and the abdominal cavity, respectively.

Since sodium restriction regularly produces elevated levels of circulating aldosterone it might be expected to produce potassium depletion, a cardinal finding in cases in which excessive aldosterone arises from tumors (27, 28). An analogous question arises in regard to these clinical states (cardiac failure, cirrhosis with ascites, and nephrosis) in which large amounts of urinary aldosterone have been repeatedly found (5, 22–26) but in which potassium depletion is an exceptional finding. For the disease states this has been explained (29) on the assumption that steroids can promote potassium excretion only by accelerating renal tubular exchange of potassium for sodium at a distal site. If, owing to complete reabsorption more proximally, virtually no sodium is available for such an exchange, aldosterone cannot increase urinary potassium (3).

The frequent finding in the cardiac and cirrhotic patient, as in the sodium-depleted normal subject, of essentially sodium-free urine is consistent with such an explanation; in all these conditions the efficiency of sodium reabsorption may be enhanced, not only by the larger amounts of circulating aldosterone, but also by decreases of filtration rate (30, 31) and thus of filtered sodium. In the patients with aldosterone-producing tumors normal filtration rates have been reported (27, 28). It appears likely that rigid sodium restriction would prevent the potassium loss in such patients (3). Furthermore, in view of the fact that patients with primary aldosteronism have been reported to excrete relatively little aldosterone (27, 28) compared to salt-depleted normal subjects or patients with cardiac failure and cirrhosis, it is likely that cases will be found in which, under the influence of much larger amounts of aldosterone, sodium retention will be almost quantitative, and edema, rather than alkalosis and potassium loss, will be the cardinal manifestation.

SUMMARY AND CONCLUSIONS

Studies were carried out in man to define the role of fluid volume in controlling aldosterone secretion.

It was shown that changes in aldosterone secretion could be effected by changes in body fluid volume, independent of changes in extracellular or intracellular ion or water concentrations.

Decreases of aldosterone secretion were produced by measures which increased extracellular fluid volume, regardless of whether intracellular water was increased (by administration of Pitressin® and water), decreased (by administration of hypertonic saline) or left unchanged (by administration of normal saline). Serum sodium concentration and osmolality fell, rose, and remained unchanged, respectively, in the three types of study.

Increases of aldosterone secretion were produced by measures which decreased extracellular fluid volume, regardless of whether intracellular water was decreased (by dehydration) or left unchanged (by administration of mercurial diuretics). Serum sodium concentration and osmolality rose, and remained unchanged, respectively, in the two types of experiment.

Changes of circulating and total body potassium could be excluded as mediators of the volume control of aldosterone secretion.

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A LABORATORY STUDY OF THE CARRIER STATE IN CLASSIC HEMOPHILIA [1]

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For many years it has been recognized that the hemorrhagic disorder now known as hemophilia is a hereditary disease of males transmitted to them only through females. From both the sociologic and medical points of view it would be important to detect those females who are conductors of the genetic defect responsible for this disease. On genetic grounds three groups of individuals can be assumed to be carriers, firstly, all daughters of a hemophiliac, secondly, the mother of two or more hemophiliacs, and thirdly, the mother of a single hemophiliac when she has other hemophilic relatives. The problem which cannot be answered on the basis of genetic hypothesis is which other female relatives of a hemophiliac are conductors. The published reports of studies of this problem are numerous and contradictory. It is the consensus that female carriers are usually asymptomatic, but there is considerable difference of opinion as to whether their blood has abnormalities detectable in the laboratory. It has only been within the last few years that the syndrome of hemophilia has been differentiated from Christmas disease (deficiency of plasma thromboplastin component). Possibly, the conflicting opinions concerning the detection of carriers have been due in part to the confusion of hemophilia with Christmas disease or with other hemorrhagic disorders. The present report concerns attempts to detect the carrier state in classic hemophilia by laboratory techniques. Among 19 presumptive and 8 possible carriers, an abnormally low concentration of antihemophilic factor was detectable in the plasma in only one instance.

MATERIALS

Blood was drawn from the antecubital veins of carriers, suspected carriers, patients with classic hemophilia, and normal adult humans, using No. 18 gauge needles coated with tris (2 hydroxyethyl) dodecyl-ammonium chloride (Monocote, Armour) and glass syringes coated with silicone (G.E. Dri-Film).

Oxalated and *citrated plasma* were prepared by mixing nine parts of venous blood with one part of 0.1 M sodium oxalate or 3.8 per cent (0.13 M) trisodium citrate dihydrate, and removing the cells by centrifugation at 2500 rpm for 15 minutes. This plasma was kept in ice water and used within a few hours, or stored at −25° C for as long as 3 months until used.

Barium sulfate-adsorbed plasma was prepared by adding one-tenth to one-fifth volume of powdered barium sulfate (Baker) to oxalated plasma which was either fresh or had been stored for as long as 1 month at −25° C. The mixture was stirred thoroughly, incubated at 37° C for 10 minutes, and then centrifuged. The supernatant barium sulfate-adsorbed plasma was kept in ice water and used within a few hours, or stored at −25° C for as long as 3 months. There was slight to moderate loss of anti-hemophilic activity in such plasma stored at −25° C for 3 months. However, in individual experiments, oxalated plasmas of similar ages were used.

Aluminum hydroxide-adsorbed plasma ("alumina-treated plasma") was prepared in the same way by adding one-tenth volume of aluminum hydroxide gel (Amphojel® Without Flavor, Wyeth) to citrated plasma which was either fresh or had been stored for as long as 3 months at −25° C, incubating the mixture at 37° C for 3 minutes, and separating the adsorbed plasma by centrifugation. The "alumina-treated plasma" was kept in ice water and used within a few hours, or stored at −25° C for as long as 8 months after the blood was drawn. In individual experiments, citrated plasmas of similar age were used although there was no detectable loss of anti-hemophilic activity in such plasma stored at −25° C for as long as 8 months. These plasmas, adsorbed with barium sulfate or aluminum hydroxide were deficient in prothrombin, proconvertin, and Christmas factor (plasma thromboplastin component).

Platelets were prepared by mixing 9 parts of blood and 1 part of 3.8 per cent trisodium citrate dihydrate in a silicone-coated lusteroid tube and separating the great bulk of the red cells and leukocytes by centrifugation at 1,000 rpm for 20 minutes at room temperature. The supernatant plasma was removed with a silicone-coated glass dropper, transferred to another silicone-coated lusteroid tube, and centrifuged at 3,000 rpm for 20 minutes. The *platelet-poor citrated plasma* was decanted and stored at −25° C. The sedimented platelets were sus-

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pended with 0.15 M sodium chloride solution, recentrifuged, and the process of washing then repeated. Finally the platelets were suspended in a volume of 0.15 M sodium chloride one-third to one-fifth of the volume of the plasma from which they were separated. The platelet suspension was stored in ice water and used within a few hours. Alternatively, the pellet of sedimented platelets was frozen without resuspension in sodium chloride solution, and stored at −25° C for as long as 5 days before use; it was then suspended in 0.15 M sodium chloride solution.

The *buffer* used consisted of 7.30 gm. of sodium chloride, 2.76 grams of barbital and 2.06 grams of sodium barbital, diluted to a volume of 1 liter with distilled water, and was thus composed of 0.025 M barbital and 0.125 M sodium chloride at pH 7.5. Centrifugation was done at room temperature in an International SB Size 1 centrifuge. Unless otherwise stated, Pyrex tubes (internal diameter 8 mm.) were used.

METHODS

The *clotting time of whole blood* was measured by a modification of the method of Lee and White (1), using dry Pyrex or silicone-coated tubes (internal diameter, 11 mm.) (2). During the period of this study, the clotting time at 25° C in Pyrex tubes averaged 27.6 minutes (range, 19 to 38; standard deviation, 4.8 minutes) and in silicone-coated tubes averaged 131 minutes (range, 75 to 185; standard deviation, 26.5 minutes), in 36 normal individuals. The silicone clotting times are slightly longer, on the average, than our overall experience with this test, and seem to reflect an undefined change in our technique. Measurement of the clotting time at 25° C and in tubes wider than those originally used by Lee and White (1) prolongs the clotting time compared with conventional methods and exaggerates the difference between normal and abnormal bloods. These modifications increase the ease of detecting minor abnormalities in the clotting mechanism.

The *serum prothrombic activity* was measured in triplicate by a technique slightly changed from one previously reported (3). A volume of 2.25 ml. of blood was allowed to clot at 37° C in Pyrex tubes. One hour after the blood was drawn, a volume of 0.25 ml. of 0.1 M sodium oxalate solution was added, and the contents mixed thoroughly with a wooden applicator stick. After incubation at 37° C for 30 minutes to destroy any remaining free thrombin, the mixture was centrifuged and the serum removed. Using Alexander's modification (4) of the method of Owren and Aas (5), the prothrombic activity of the patient's serum was then compared with that of oxalated plasma which had been incubated for the same period of time. In 17 normal individuals, the serum prothrombic activity ranged from less than 10 per cent to less than 25 per cent of plasma.

The *anti-hemophilic activity* of barium sulfate-adsorbed oxalated plasma and of aluminum hydroxide-adsorbed citrated plasma was estimated by determining the plasma's capacity to correct the abnormality of plasma which was known to be deficient in anti-hemophilic factor, *i.e.*, hemophilic plasma. The plasma to be tested was diluted to the desired concentration with buffer. In duplicate, 0.1 ml. of hemophilic plasma, 0.1 ml. of the diluted test plasma, and 0.1 ml. of 0.025 M calcium chloride solution were mixed in Pyrex tubes immersed in ice water. The clotting time was then determined in a water bath at 25° C by tilting the tubes at approximately 30-second intervals. The clotting time was the period which elapsed from the time that the tubes were transferred to the 25° C bath until the appearance of a clot. The longer of the duplicate clotting times is recorded. When the clotting time was 12 minutes or less, the plasma in duplicate tubes usually clotted within one-half minute of each other. When the clotting time was 20 minutes or less, the plasma in duplicate tubes almost always clotted within one minute of each other.

The thromboplastin generation test was performed according to the method of Biggs and Douglas (6). Platelet solutions were used at either 3, 4, or 5 times the original plasma concentration. In one experiment the aluminum hydroxide-adsorbed citrated plasma was diluted with 39 volumes of 0.15 M sodium chloride solution instead of with 4 volumes, as originally recommended.

To assay for the presence of a *circulating anticoagulant*, 9 parts of the citrated plasma to be tested were incubated in Pyrex tubes with one part of normal citrated plasma at 37° C for 3 hours. At the same time, portions of the normal plasma and the plasma to be tested were incubated separately for 3 hours at 37° C and at the end of this time were mixed in the same proportions. Each mixture was then diluted with 19 volumes of buffer. A volume of 0.1 ml. of each dilution was mixed with 0.1 portions of hemophilic plasma and 0.1 ml. of 0.025 M solution of calcium chloride, and the recalcified clotting time measured at 25° C in Pyrex tubes. This test detects circulating anticoagulants not demonstrable by more usual techniques. A circulating anticoagulant has been demonstrated in 5 of 22 hemophiliacs studied in this laboratory.

RESULTS

The diagnosis of classic hemophilia was established in at least one male member of each family in the usual manner. In no instance was the plasma of one patient capable of correcting the defect in the plasma of another patient. Several of these hemophilic plasmas were tested for their ability to correct the defect in patients with Christmas disease (plasma thromboplastin component deficiency). In each instance there was full correction.

In all, twenty-seven carriers in eighteen families were studied (Table I). The carriers are numbered 1 through 27, and the families are numbered with Roman numerals (I through XVIII).

TABLE I

The clotting times and serum prothrombic activity of 26 carriers

Carriers*	Families	Normal range:	Glass clotting time (*min.*) 19–38	Silicone clotting time (*min.*) 75–185	Serum prothrombic activity (% *of plasma*) <25
1	I		26	125	<10, <10, <10
2	I		30	115	10, 10, 17
3	I		24	115	<10, <10, 15
4	I		25	125	<10, <10, 11
5	II		30	215	10, 13, 17
6	II		28, 33	>205, 175	11, 15, 20
7	III		28	155	17, 17, 22
8	II		24, 26	115, 115	10, 11, 13
9	II		27	135	<10, <10
10	IV		28, 23	135, 145	<10, <10, 12
11	IV		25	125	<10, <10, <10
12	IV		22	175	<10, <10, <10
13	V		27	155	10, 10, 12
14	V		32	115	<10, <10, <10
15	VI		29, 30	145, 205	11, 11, 12
16	VII				
17	VIII		25	155	12, 13, 14
18	IX		29	155	13, 13, 14
19	X		29	205	<10, <10, <10
20	XI		27	105	14, 19, 20
21	XII		21	75	10, 13, 14
22	XIII		30	155	<10, <10, <10
23	XIV		34	145	<10, 10, 11
24	XV		28	145	<10, <10, 12
25	XVI		22	115	<10, 10, 16
26	XVII		27	145	10, 10, 13
27	XVIII		24	125	11, 12, 15

* The same numbers are used to designate carriers in all subsequent tables.

Carriers 1 through 7 are daughters of hemophilic males. Carriers 8 through 19 are also genetically established carriers because of the presence of hemophilia in one or more sons and collateral lines. In contrast, carriers 20 through 27 have only one hemophilic son each; no additional familial bleeding history could be obtained. Thus carriers 1 through 19 are presumptive conductors, and carriers 20 through 27 are possible conductors of hemophilia. The term *carrier* will be used to denote both the presumptive and the possible carriers.

The clotting times in glass and in silicone-coated tubes of 26 carriers are shown in Table I. Clotting times of 4 carriers were determined on two separate occasions. In all cases the clotting times of blood in glass tubes were within normal limits. Slightly prolonged clotting in silicone-coated tubes occurred once each in carriers 5, 6, 15 and 19.

The residual serum prothrombic activity (Table I) was determined usually in triplicate, on one occasion in each of twenty-six carriers. All the determinations were within normal limits.

The thromboplastin generation test was performed in six experiments. Aluminum hydroxide-adsorbed citrated plasmas of 26 carriers and 28 normal controls were tested. In 17 instances the carriers were studied in at least 2 separate experiments. To increase the sensitivity of the test in detecting abnormal alumina plasma, the alumina-treated plasma was diluted 1 to 40 instead of 1 to 5 (Figure 1). Using the same normal platelets and normal serum throughout, the diluted plasmas of 13 carriers and 10 controls were tested. The ranges of the clotting times of platelet-poor citrated plasma are shown. There was no significant difference in the behavior of alumina-treated plasma of the carriers and of the controls.

In order to increase its sensitivity in detecting abnormal alumina plasma, the thromboplastin generation test was modified in another way in 3 experiments. Normal platelets and normal serum were used throughout, but the alumina plasma consisted of 4 parts of hemophilic alumina-treated plasma and 1 part of the test alumina-treated plasma; the mixture was diluted 1 in 5. In 1 of these experiments the alumina-treated plasma of

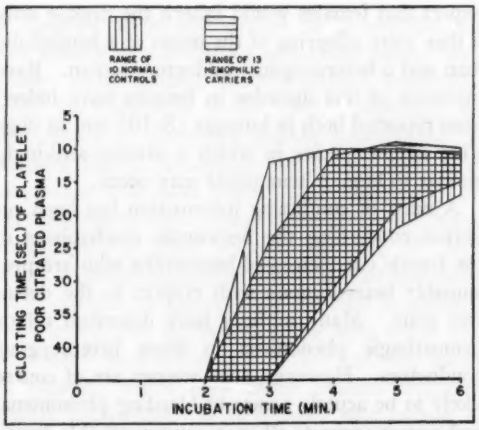

FIG. 1. THE THROMBOPLASTIN GENERATION TEST IN HEMOPHILIC CARRIERS

Equal volumes of 1) aluminum hydroxide citrated plasma, diluted 1/40, 2) normal pooled serum, diluted 1/10, 3) normal platelets concentrated 5 times, and 4) 0.025 M calcium chloride solution were incubated at 37° C in Pyrex tubes (internal diameter 11 mm.). At intervals of one minute beginning with the second minute 0.1 ml. aliquots of this "incubating mixture" and 0.1 ml. of 0.025 M calcium chloride solution were added to 0.1 ml. of platelet-poor citrated plasma, and the clotting time determined.

carrier 6 produced the longest clotting times of platelet-poor plasma. Otherwise there was no apparent difference between the carrier and the control groups.

In another experiment "thromboplastin generation" was measured in the manner just described, but hemophilic platelets were used instead of normal platelets. There was no appreciable difference in the clotting times of platelet-poor plasma upon the addition of an incubation mixture containing normal aluminum hydroxide-adsorbed plasma or aluminum hydroxide-adsorbed plasma prepared from carriers.

In another experiment the test was performed in the manner described, using normal platelets and normal serum. The activity of the alumina plasma of the 4 carriers of family II (including carrier 6) was compared with that of the normal controls. There was no appreciable difference in the clotting time of platelet-poor plasma upon the addition of incubating mixture containing normal alumina-treated plasma or alumina-treated plasma prepared from carriers.

Anti-hemophilic activity may be assayed more specifically by testing the ability of diluted barium sulfate-adsorbed oxalated plasma ("test plasma") to correct the prolonged recalcified clotting time of hemophilic plasma. By comparing the clotting times recorded for various dilutions of the test plasmas, one can compare their anti-hemophilic activities. Nineteen separate experiments were performed with the plasmas of 21 carriers and of 26 normal controls. A typical experiment is shown in Table IIA. The anti-hemophilic activity of the plasmas of carrier 4 and of carrier 20 was at least equal to that of the 2 normal controls. It is of interest that the plasma of the first normal control has twice the anti-hemophilic activity of the plasma of the second control. Occasionally by this technique as great as a three-fold difference in anti-hemophilic activity may be demonstrated among normal plasmas. Therefore, when the anti-hemophilic activity of a carrier's plasma was not as great as that of any one of 3 or 4 control plasmas, the carrier's plasma was compared with a pool of 4 to 6 normal plasmas. If the plasma of the carrier had at least half the anti-hemophilic activity of the normal pooled plasma, the carrier's plasma was not considered to be significantly deficient in anti-hemophilic activity. Of the 21 carriers tested, only the barium sulfate-adsorbed plasma of carrier 6 was grossly deficient in anti-hemophilic activity (Table IIB), having only one-fourth the corrective effect of normal control plasma or of pooled normal plasma.

TABLE II

Assay for anti-hemophilic activity in barium sulfate-adsorbed oxalated plasma *

		Initial dilution of test plasma			
A.	Test plasma	1/5	1/10	1/20	1/∞†
	Carrier No. 4	8	9	10	17
	Carrier No. 20	8	10	11	
	Normal control	8	9	10	
	Normal control	9	10	11	
B.	Carrier No. 6	12	13	14	18
	Normal control	10	10	11	
	Normal control	9	11	11	
	Normal control	10	10	12	

* *Clotting time* (min.) at 25° C in Pyrex tubes containing 0.1 ml. each of 1) hemophilic citrated plasma, 2) barium sulfate-adsorbed oxalated plasma of person to be tested, diluted with buffer as indicated, and 3) 0.025 M solution of calcium chloride.

† Buffer.

TABLE III

Assay for anti-hemophilic activity in aluminum hydroxide-adsorbed citrated plasma *

		Initial dilution of test plasma			
A.	Test plasma	1/5	1/10	1/20	1/∞†
	Carrier No. 27	8	9	10	18
	Normal control	8	9	11	
	Normal control	8	10	11	
	Hemophiliac	18	19	18	
B.	Carrier No. 6	10	12	12	18
	Normal control	8	9	10	
	Normal control	8	9	10	

* *Clotting time* (min.) at 25° C in Pyrex tubes containing 0.1 ml. of 1) hemophilic citrated plasma, 2) aluminum hydroxide-adsorbed citrated plasma of person to be tested, diluted with buffer as indicated, and 3) 0.025 M calcium chloride solution.

† Buffer.

Similarly, the ability of aluminum hydroxide-adsorbed citrated plasma of 21 carriers (including the six whose barium sulfate-adsorbed plasmas were not tested) and of 19 normal individuals to correct the prolonged recalcified clotting time of hemophilic plasma was compared in 16 separate experiments. A typical experiment (Table IIIA) shows that the anti-hemophilic activities of the alumina-treated plasma of carrier 27 and of the two normal controls are roughly similar, but that the alumina-treated plasma of a classic hemophiliac is completely deficient in anti-hemophilic activity. Of the 21 carriers tested by this technique, only the alumina-treated plasma of carrier 6 was grossly deficient in anti-hemophilic activity (Table IIIB), having only one-fourth the corrective effect of normal control plasma or pooled normal plasma.

It has been suggested that the inability of the plasma of a carrier to correct the hemophilic defect may be due to the presence in that plasma of a circulating anticoagulant which actively destroys the anti-hemophilic substance (7). The plasma of carrier 6 did not inactivate the anti-hemophilic factor of normal plasma even after prolonged incubation, and presumably did not contain a detectable circulating anticoagulant.

DISCUSSION

Characteristically, hemophilia is a disorder limited to males and inherited in a manner suggesting that the defect is transmitted by a sex-linked recessive gene. On theoretic grounds, one would expect that females would inherit the disease only if they were offspring of the union of a hemophilic man and a heterozygous conductor woman. Rare instances of this disorder in females have indeed been reported both in humans (8–10) and in dogs (11, 12), a species in which a disease strikingly similar to human hemophilia may occur.

A mass of conflicting information has been reported concerning the hemostatic mechanisms in the female conductors of hemophilia who are presumably heterozygous with respect to the defective gene. Many authors have described minor hemorrhagic phenomena in these heterozygous conductors. However, such women are of course likely to be acutely aware of bleeding phenomena, and may exaggerate otherwise unremarkable symptoms. It is not as easy to dismiss the many reports of abnormalities detectable in the laboratory. Among the coagulative defects which have been described in hemophilic carriers are prolonged whole blood clotting times (13–21), abnormal "utilization" of prothrombin during clotting (22–28), diminished activation of proconvertin and formation of proaccelerin during clotting (29), diminished "tolerance" of whole blood to heparin (30) and abnormal thromboplastin generation tests (30). It has also been reported that the plasma of carriers will not correct the defect in hemophilic plasma as readily as will normal plasma (30, 31).

An equally impressive array of data have been published supporting the view that the blood of hemophilic carriers does not contain detectable hemostatic abnormalities. Thus it has been stated that the blood of carriers has a normal clotting time in glass (26, 30, 32–35) or in silicone-coated (31) tubes, and a normal "tolerance" to heparin (26), and significantly shortens the clotting time of hemophilic blood (33, 35). The "consumption" of prothrombin during clotting (30, 31, 33, 35, 36) and thromboplastin generation tests (3, 37) are said to be normal.

There are at least two possible explanations for these discordant results. In the first place, the various authors may not have been studying the same disease in their various patients. It has only recently been appreciated that the symptom complex originally termed hemophilia might result from a variety of abnormalities in the plasma. Thus, several groups of investigators described

a disorder strikingly similar to hemophilia but due instead to a deficiency of another factor, variously called plasma thromboplastin component (38) or Christmas factor (39). Although the inheritance of Christmas disease superficially resembles that of hemophilia, the female carriers of the former may have hemorrhagic symptoms (26, 40) and evidences of a partial deficiency of Christmas factor in their plasma (26, 37, 39, 40). More recently, Rosenthal, Dreskin, and Rosenthal (41) observed a hemorrhagic disorder resembling mild hemophilia but occurring in both sexes, and attributed to a deficiency of yet another factor in plasma, which they called plasma thromboplastin antecedent. It is possible that certain of the reports in which the identification of the carrier state was described may have concerned patients with Christmas disease or plasma thromboplastin antecedent deficiency. However, it must be remembered that most patients with hemophilia-like disease do have classic hemophilia. Our own experience, which now includes 28 families with classic hemophilia but only 6 families with Christmas disease, is typical of data which have been reported by others. This would suggest that misdiagnosis plays at most a small part in the contradictory results which have been reported.

A second explanation for the variation in opinion concerning the detection of hemophilic carriers may reside in the technical methods used. For example, many of those who have claimed that the clotting time of the blood of hemophilic conductors is prolonged used the method of Bürker (42). In this test, the end-point is more subjective than in the Lee-White (1) technique. In the last analysis, the only tests which are specific for a deficiency in anti-hemophilic factor are those in which the capacity of the unknown plasma to correct the defect of known hemophilic plasma is measured. Such non-specific tests as the clotting time or the prothrombin consumption test may be abnormal in any given subject because of quite irrelevant factors. Indeed, the converse is often true, namely, that these tests may give normal results in classic hemophiliacs (43–50). However, any abnormality present in a hemophilic carrier might be manifested by a true decrease in the plasma's anti-hemophilic activity, were the defective gene "incompletely recessive."

In the present study, a significant reduction in anti-hemophilic activity compared with normal plasma was noted in the plasma of only one carrier, No. 6. She is the asymptomatic 9-year-old daughter of a true hemophiliac. This same young girl had a long silicone clotting time on one of two occasions. Her sister, No. 5, had a long silicone clotting time, but assays of her plasma did not clearly demonstrate a deficiency of anti-hemophilic factor. Two other proved carriers in this family had no demonstrable abnormalities, although they bruised easily and bled after dental extractions. The two known hemophiliacs in this family bled only occasionally, and although hemarthroses were observed in one, no crippling deformities ensued. However, the father of carriers No. 5 and No. 6 bled to death after exploratory laparotomy.

The data we have presented make it appear unlikely that with the laboratory techniques presently available a significant percentage of female carriers in families with classic hemophilia can be identified by a study of the concentration of anti-hemophilic factor in their blood. It is of interest that in the one family in which it was possible to demonstrate any changes, the hemophilia appeared to be relatively mild. This observation is of particular interest because of the study by Graham, McLendon, and Brinkhous (51) of a family of classic hemophiliacs in whom the disorder was mild. None of their patients had suffered a hemarthrosis. Graham, Collins, Godwin, and Brinkhous (52) and Langdell and Wagner (53) assayed the plasma of 10 (presumed or possible) carriers (in this family) by a method specific for anti-hemophilic factor, and demonstrated that 6 of the 10 had concentrations of 31 to 54 per cent of normal, values which they considered clearly pathologic.

Similarly, the Moena family, originally reported to have a unique disorder, was also noteworthy because of the association of mild hemophilia (54) and detectable changes in the blood of female carriers (23). These families, then, resemble that of carrier No. 6 in the present report.

Although the hemophilia was mild in those families in which carriers were detected, this may be fortuitous. Lewis (55) has observed a significantly low titer of anti-hemophilic activity in 7 of 40 presumed or possible carriers. In her series there did not appear to be any correlation between

the severity of the disease and the anti-hemophilic titer in the female carriers.

Because the clinical severity of hemophilia varies in different families, and because of the abnormalities found in the blood of carriers of mild hemophilia, it has been suggested that the genetic transmission of hemophilia is not simply dependent upon the presence or absence of a single recessive gene. Rather it has been suggested that there is a series of isoallelic genes (51), each manifested clinically by hemophilia of a given severity, and each associated with a corresponding level in the blood of anti-hemophilic factor. An alternate but basically similar explanation is that a single recessive gene is responsible for the disease, but that there are also modifying genes (37, 56) which alter its clinical expression. The data which have been presented do not lend themselves to the support of one or another of these hypotheses.

SUMMARY

The medical literature relating to the detection of hemophilic conductors by laboratory techniques is conflicting. Routine clotting studies and assays for anti-hemophilic factor were performed in 27 carriers of classic hemophilia. A definitely reduced concentration of anti-hemophilic factor was demonstrated in only one carrier, although minor abnormalities of the clotting mechanism were found in several others. In the families of hemophiliacs, it is possible to detect only a small number of carriers of the trait with currently available techniques.

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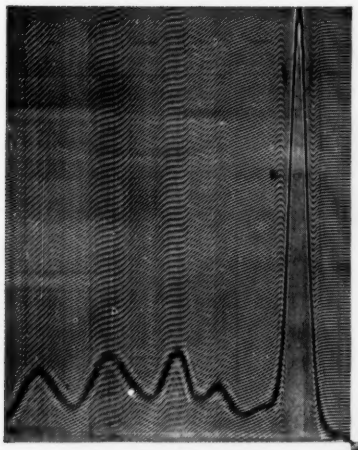
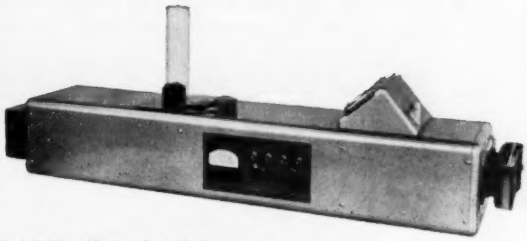


Ascending branch of albumin peak.



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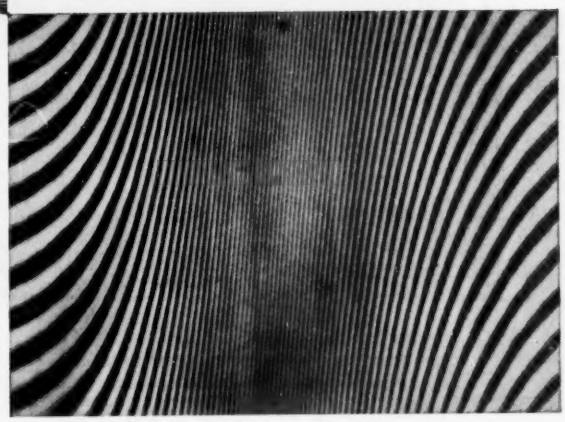

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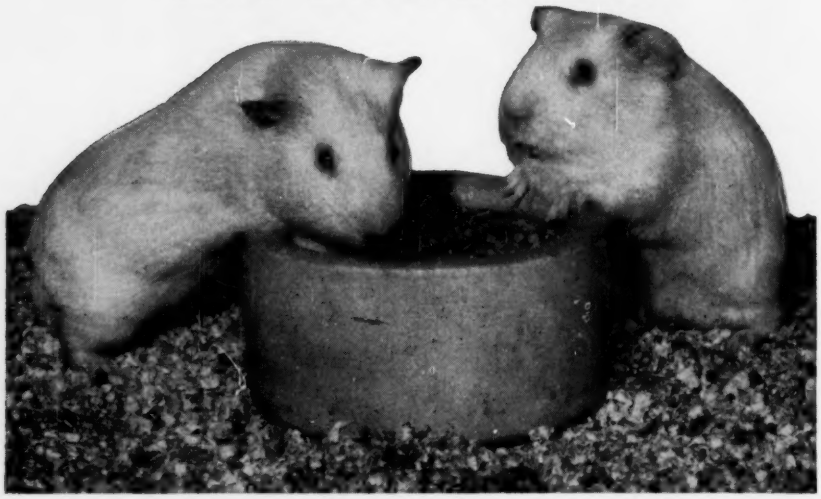

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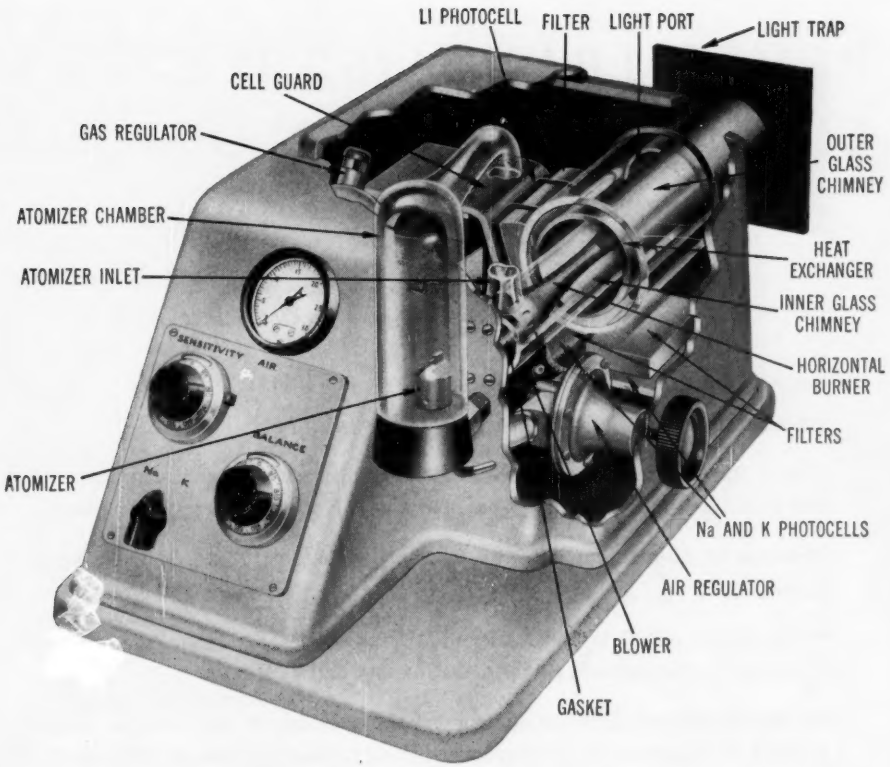

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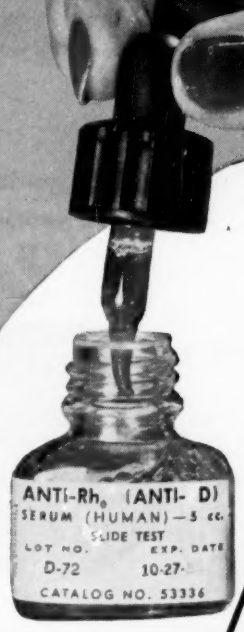

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